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in PLANTS

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PHOTOSYNTHESIS IN PLANTS

*A Monograph of
The American Society of
Plant Physiologists*

EDITED BY

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PROFESSOR OF CHEMISTRY
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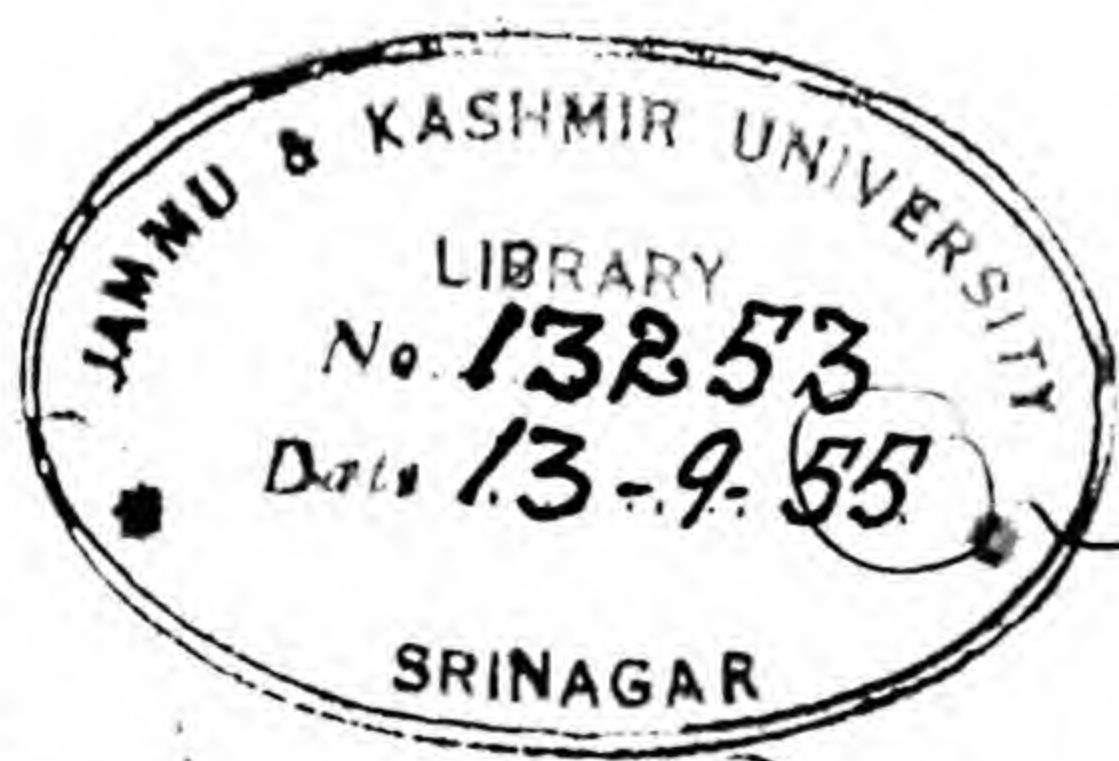
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Preface

THE MONOGRAPH BOARD of the American Society of Plant Physiologists is pleased to be able to offer Photosynthesis in Plants as the second volume of its Monograph Series. Photosynthesis is pre-eminently a plant physiological topic, yet, it is at the same time of basic interest to Organic and Biochemists and to all Biologists.

The Board is glad to acknowledge its indebtedness to Section C of the American Association for the Advancement of Science, and particularly to Drs. Neil E. Gordon and Farrington Daniels. The present volume has grown out of the Gibson Island Conference on Photosynthesis organized by Dr. Gordon in 1941. Following that meeting Dr. James Franck was asked to serve as senior Editor and invitations were sent to most of the prominent American workers in the field to collaborate on a monographic treatment of photosynthesis.

The war interrupted plans for publication in 1943. After the war various workers wished to expand their research before attempting reports, so that plans were proceeding slowly when Dr. Daniels organized a Section C Symposium on Photosynthesis for the Chicago meetings. Since many of the same persons were preparing papers for the Monograph and the Symposium, Dr. Daniels kindly permitted the Editors of the Monograph to invite publication of the Symposium papers. With the exception of one or two manuscripts, for which previous commitments had been made, the record of the Chicago Symposium is included in the present volume along with the review articles prepared especially for this publication. The Board is deeply grateful to Dr. Daniels and to each of the Authors without whose cooperation this book would have been impossible.

We are especially indebted to Dr. James Franck for the counsel and leadership which has assured the quality of the volume and its excellent coverage of a broad and ramifying subject. As Plant Physiologists we consider it one of the dividends of the recent troubled times that Dr. Franck should have been diverted into our field, and have been able to apply his talents and energy to the problems of Photosynthesis.

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The American Society of
Plant Physiologists*



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1.

Photosynthesis—An Introduction

W. E. LOOMIS¹

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Ames, Iowa*

PHOTOSYNTHESIS is widely rated as the world's most important chemical reaction. It is the sole basis of our food supply and the major factor in supplying both the raw materials and the energy of industry. Mankind has progressed from the Stone Age to the Atomic without a reduction in his dependence upon plants and their unique ability to utilize radiant energy to reduce CO_2 to biologically and industrially usable forms. Chemistry has been able to substitute coal tar compounds for indigo or rose oil, spruce-wood cellulose for cotton and silk, the products of the pine tree for those of the camphor tree, but all of these modern developments are still dependent upon some form of photosynthetically reduced carbon.

WORLD TOTALS OF PHOTOSYNTHESIS

Not only is photosynthesis the basis of our existence, it is an industry of enormous magnitude. The world production of steel is of the order of 100 million tons a year. The world production of primary photosynthate is more than 2,000 times as great.

An average acre of U. S. corn (maize) stores 0.75 short tons of carbon per acre per year (1.68 metric tons/hectare) without allowing for root production or respiration, which together would bring the figure to about one ton. Maximum corn crops fix six times this much carbon and maximum crops of sugar cane may fix more than 20 tons of carbon per acre per year. Many desert or frozen areas, however, are nearly bare of plants. The average grass meadow fixes only 0.6 tons, including an allowance for respiration, and total photosynthesis on large areas of range land uses less than 0.1 tons of carbon. Schroeder's estimate (15) of 0.5 tons average fixation of carbon per acre of the earth's land

¹We regret that illness has prevented Dr. Franck from collaborating in the preparation of this section as originally planned.

surface per year seems reasonable, therefore, and is probably as good an estimate as we have available. With a land area of 37 billion acres, our figure for total photosynthesis becomes 18.5 billion tons of carbon, or about 68 billion tons of CO_2 fixed by land plants each year. These figures are close to 10 per cent of the average carbon or CO_2 content of the air over the given area.

Estimates of the amount of photosynthesis in the ocean vary from considerably less than the land to three or more times as much. Certainly the total photosynthesis of the 300 foot layer of phytoplankton, mostly diatoms, of the nearly 90 billion acres of oceans should be more than that of land plants. Rabinowitch (14) estimates total ocean photosynthesis as eight times the land total. Even if the per acre rate in the ocean were only twice that on land, a not unreasonable assumption in view of the much higher CO_2 content of the ocean and the thick layer of plants involved, total photosynthesis in the ocean would amount to some 90 billion tons of carbon. Our annual total for the earth would then be 108,000,000,000 tons of carbon, equivalent to 270,000,000,000 tons of glucose, and requiring 396,000,000,000 tons of CO_2 .

THE CARBON CYCLE

The air above one acre of land contains something over 5 tons of carbon, or 18 to 20 tons of CO_2 , and the total carbon content of the troposphere is of the order of 600 billion tons. The oceans, however, contain nearly 100 times more (18), in the form of dissolved CO_2 and carbonates in equilibrium with the air, so that the available supply of carbon is some 50 trillion tons, enough to last 400 or 500 years at our estimated rate of total photosynthesis. The carbon of the biosphere, also, tends to return to the available form of CO_2 , in contrast to nitrogen for example, which escapes rather readily to the unavailable form of free N_2 . The decay of plants, the respiration of plants and animals, and combustion of all kinds return CO_2 to the air-water reserve. The respiration rate of plants is estimated at 15 per cent of the rate of photosynthesis, or sufficient to free some 16 billion tons of carbon each year. Animal respiration is harder to estimate, but the total carbon liberated by the world's human population is of the order of 0.3 billion tons a year. Most of the earth's animals are cold blooded with a relatively low rate of respiration so that the total for all animal respiration is probably considerably less than the figure for plants. This carbon is derived, of course, from the plants eaten, either directly or indirectly by the animals, and leaves some 80 per cent of the annual plant synthesis to be broken down by microorganisms in the processes of decay, or locally by fire.

The major paths of the carbon cycle of the troposphere are shown graphically in Figure 1.1, with the relative importance of the processes suggested by the width of the lines. Within the ocean itself a similar cycle of photosynthesis, respiration and decay occurs, with some of the reduced carbon compounds settling to the ocean

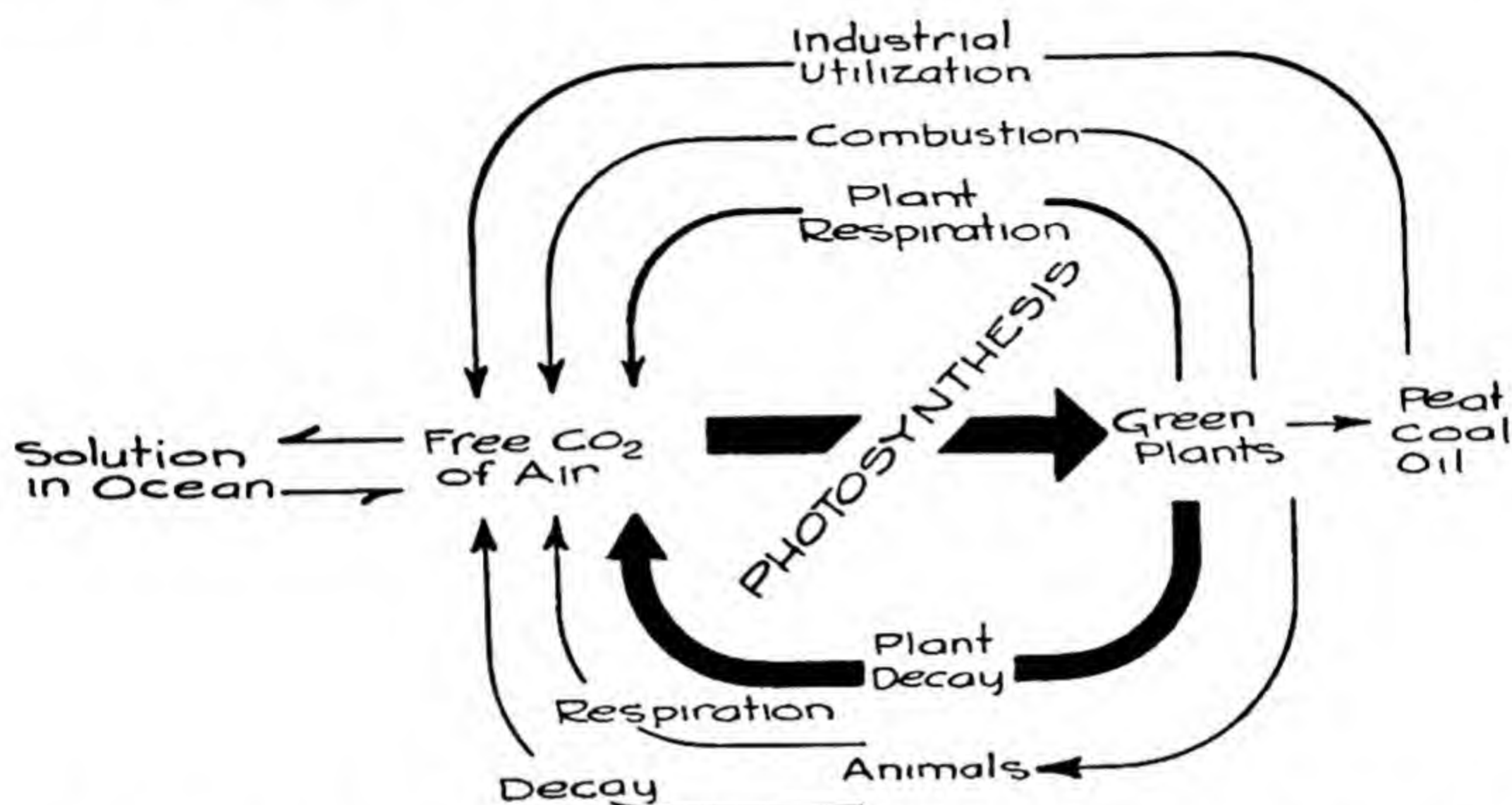


FIG. 1.1—The carbon cycle. CO₂ is removed from the air by photosynthetic plants, and largely returned by the microscopic plants responsible for decay.

floor where they may be formed into petroleum.

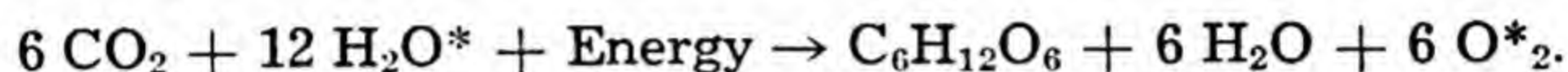
Because plant respiration and the action of the decay organisms follow the same seasonal trend as photosynthesis, and because of the tremendous buffering capacity of the oceans, the CO₂ content of the air remains at an average of 0.03 per cent by volume throughout the year. There may be sharp local fluctuations, however, with rain, photosynthesis, and other factors. The CO₂ content of the air at Ames varied in three summers from 0.02 to 0.04 per cent. Very typically the nighttime percentage was near 0.03, dropping with photosynthesis in the morning to about 0.023 per cent at 10:00 A.M., and rising again in the evening.

THE PHOTOSYNTHETIC EQUATION

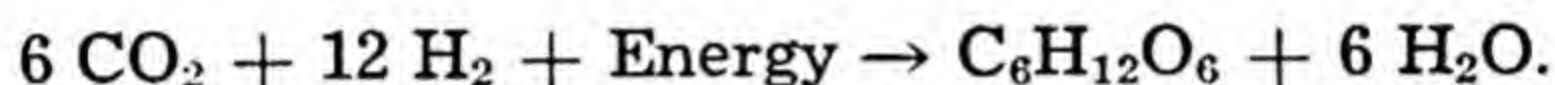
Although a number of the authors in this volume point out other probable end products (cf. Chap. 17), it is conventional and convenient to assume that a hexose sugar is the primary end product of photosynthesis. Certainly plants can synthesize, under favorable conditions, most or all of their compounds from sugar and minerals. It can be demonstrated also (10) that the common sugars

dextrose, levulose, and sucrose are so readily interchangeable in the plant that the "first" sugar cannot be determined.

The formation of a molecule of $C_6H_{12}O_6$ will obviously require six molecules of CO_2 . We have assumed in the past that the minimum six molecules of H_2O will be used. The evidence that twice this much water is actually used is discussed in Chapters 3 and 18, and implied in the photoreduction experiments reported by Rieke (Chap. 12). The question of whether six molecules of H_2O are used or twelve used and six recovered may seem academic, but the difference is basic in the establishment of the mechanism of the photosynthetic reaction. With twelve molecules of H_2O , our elementary, over-all photosynthetic equation becomes:



In photoreduction where molecular hydrogen is supplied (cf. Chap. 12) we have:



The two equations indicate that water is not combined with CO_2 to form a carbon hydrate, but that it is decomposed and its hydrogen used in the reduction of CO_2 . As we shall show below this series of reactions is not only indicated by tracer experiments with water or CO_2 containing heavy isotopes of oxygen, but is more or less required by the limitations of the energy source.

QUANTUM EFFICIENCY

If one molecular equivalent, 180 gm., of pure glucose is burned in a calorimeter, energy variously estimated as 673 to 679 kg. cal. is liberated. These figures represent the minimum of energy required for photosynthesis. Elementary experiments will show that this energy is derived from sunlight or other radiation having wave lengths between about 4,000 and 7,000 Å, that is from the visible spectrum. From other experiments we know that radiant energy can be absorbed only by specific portions of single molecules in discrete units called quanta. The absorption of one quantum of radiation by one molecule of chlorophyll increases the energy of the molecule many times, to a point which would be quickly fatal if all the molecules of the leaf were affected. This high energy level is available for the very rapid photochemical reactions of photosynthesis, but it is either used or scattered so quickly that the heat sensitive protoplasm of the leaf is not injured.

The caloric energy of a quantum of radiation varies inversely with the wave length so that a quantum of radiation at 4,000 Å carries twice the energy of one at 8,000 Å. Since we know, however,

that radiation at 6,000 Å is fully effective in photosynthesis, we can base our calculations on this wave length. If we divide the heat of combustion of glucose by the number of carbon atoms, we find that the quotient is approximately three times the energy carried by one quantum of radiation at a wave length of 6,000 Å. That is, three quanta of radiant energy will be required to change one atom of carbon from CO_2 to CH_2O , provided none of the energy is wasted or used in other reactions. Known wastage and the fact that quanta cannot be divided brings our minimum to four quanta per molecule of carbon reduced, or a quantum efficiency of 0.25 molecules of carbon per quantum. Warburg (19) claims to have demonstrated this level of efficiency.

It now becomes interesting to note that our photosynthesis equation calls for the transfer of four atoms of hydrogen in the reduction of one molecule of carbon. Each of these transfers will require energy, and the difficulties of storing radiant energy in a reactive form or of getting four quanta of radiation in one place at one time, indicate that each transfer uses one quantum, with any surplus of energy dissipated as heat. If, now, any energy is required for other purposes, such as a second endothermic transfer of hydrogen, more quanta would be necessary.

The evidence that the quantum efficiency of photosynthesis is 0.08, 0.10 or at most 0.125 instead of the theoretically possible 0.25, is presented and discussed in Chapters 10 through 13. The preponderance of the evidence, obtained in a number of different ways, indicates that at least eight quanta are required per molecule of carbon, so that two radiant energy transfers of each atom of hydrogen become possible. Rieke's report (Chap. 12) that the quantum efficiency was the same whether H_2O or H_2 was used as a source of hydrogen indicates that hydrogen transfers rather than the simple decomposition of water are involved in the first energy consuming step, and we can speculate on the possibility that one quantum of energy is used to transfer hydrogen to an acceptor (chlorophyll?) whether or not a concurrent decomposition of water is required, and a second to transfer the same atom of hydrogen to the CO_2 complex.

THE ABSORPTION OF ENERGY BY LEAVES

The green color of leaves is due to the greater absorption of the shorter and longer wave lengths of the visible spectrum, and has given rise to the general assumption that the wave lengths of the green region are of little value in photosynthesis. If the acetone soluble pigments from unit area of a typical green leaf are exposed in an absorption cell having an equivalent area, the

absorption at 5,500 Å (green) is of the order of 20 per cent, while that at 6,600 Å may be more than 90 per cent. A number of workers (3, 13, 16) have shown that the same quantity of pigment in an intact leaf gives a very different spectrum. The transmission spectra of two leaves and of solutions of their pigments at the same concentration per unit area are shown in Figure 1.2. The curves show three times as much green light ($0.55\ \mu$ or 5,500 Å) transmitted by the ether solutions as by the leaves. Because the leaves reflect approximately as much light as they absorb, however, while solutions do not, the figures indicated for absorption are 25 per cent for the pigment solutions in ether and 50 per cent for the same pigments in the intact leaves. Rabideau, French, and Holt (13) found about 50 per cent absorption of the green light by a number of green leaves tested in an Ulbricht sphere, with leaves of *Ficus* going above 80 per cent. Seybold and Weissweiler (16) claim 80 to 90 per cent for typical leaves and the data of Figure 1.3, which are the average of measurements on ninety species, show 80 per cent absorption. Reflections here were measured in a Razeek-Mulder spectrophotometer and transmissions by pressing the leaf close against a Weston photronic cell, to reduce losses of scattered light, and irradiating with a monochromator.

The transmission spectra of the normal yellow green and the nearly white portions of a variegated leaf of *Coleus blumei* are shown in Figure 1.4. Since leaves scatter the unabsorbed radiation nearly equally in all directions, a transmission of near 50 per cent indicates zero absorption. The absorption of blue light by the "white" leaf indicates the presence of yellow pigments. The differences between the two curves may be assigned to absorption by pigments, particularly chlorophyll.

Reference to Figures 1.2 and 1.4 shows that transmission in the far red was approaching the 50 per cent point indicating zero absorption. Unpublished work by Dinger (3) shows a minimum absorption by leaves in the near infrared at 8,000 Å or $0.8\ \mu$. Between 1 and $3\ \mu$ absorption increased steadily, with absorption bands due to water at 1.4 and $1.9\ \mu$. More unpublished research done by the writer with Professor H. M. Randall at the University of Michigan shows that absorption of far infrared by even a thin, moist leaf becomes complete shortly beyond $3\ \mu$ and between this point and $17\ \mu$ or more. None of these wave lengths are used in photosynthesis so that their physiological effects are indirect. The low absorption of active leaves in the near infrared, which constitutes a considerable portion of sunlight, reduces heating of the leaf. Complete absorption of the wave lengths radiated by

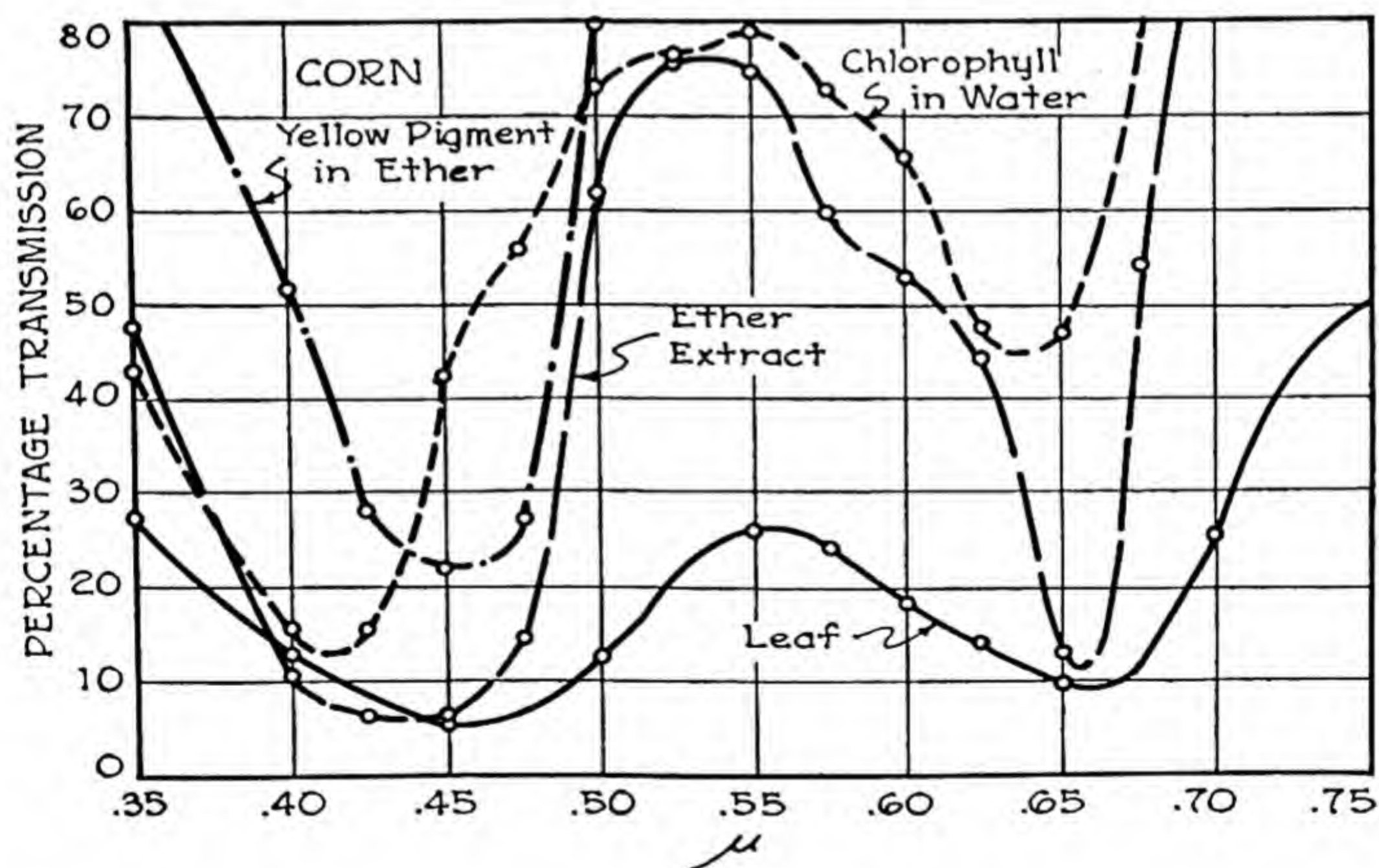
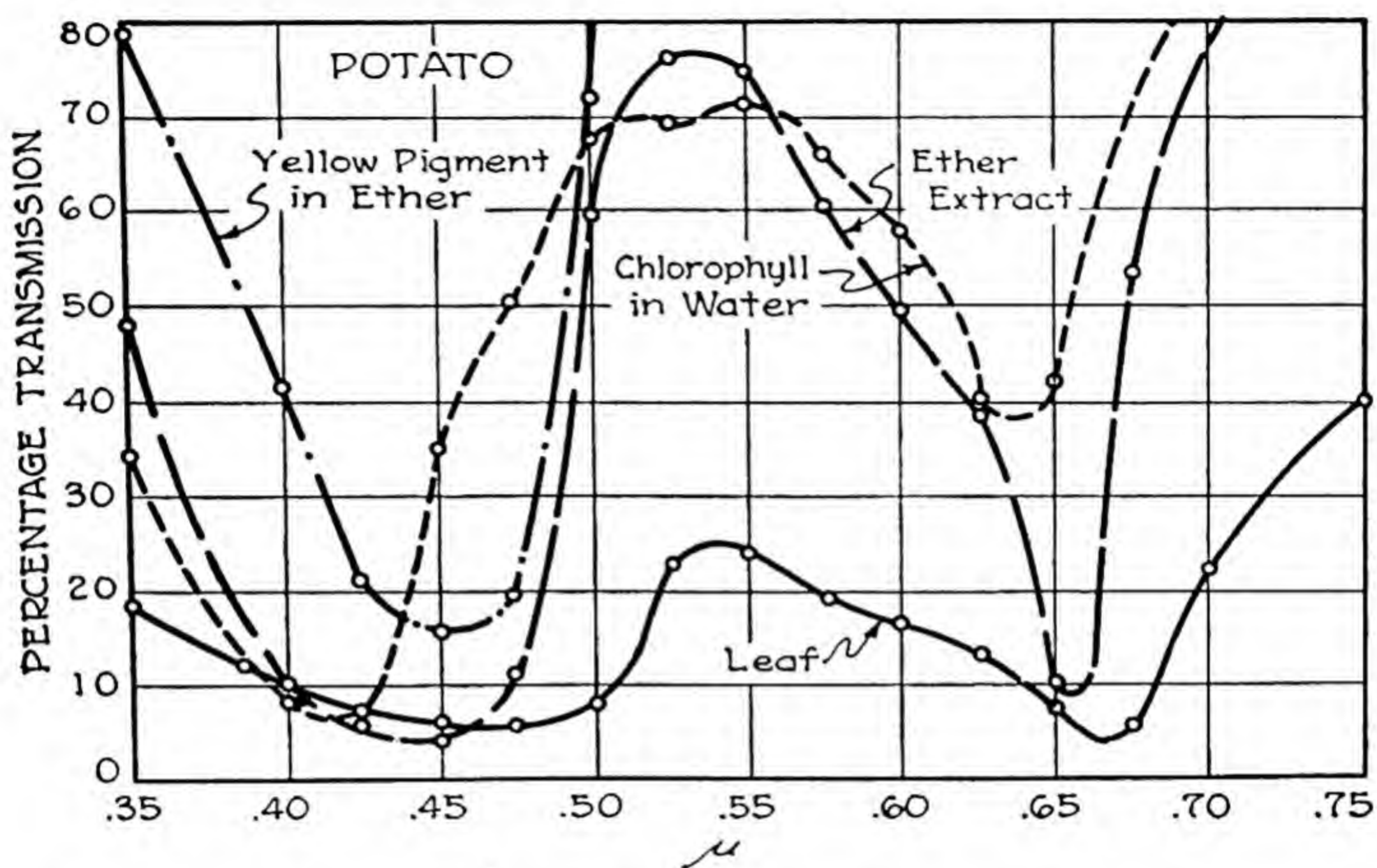


FIG. 1.2—The transmission spectra of leaves and of equivalent quantities of pigments in solution. Ether extracts of the leaf pigments absorb somewhat more blue and red wave lengths but only half as much green as the same pigments in leaves.

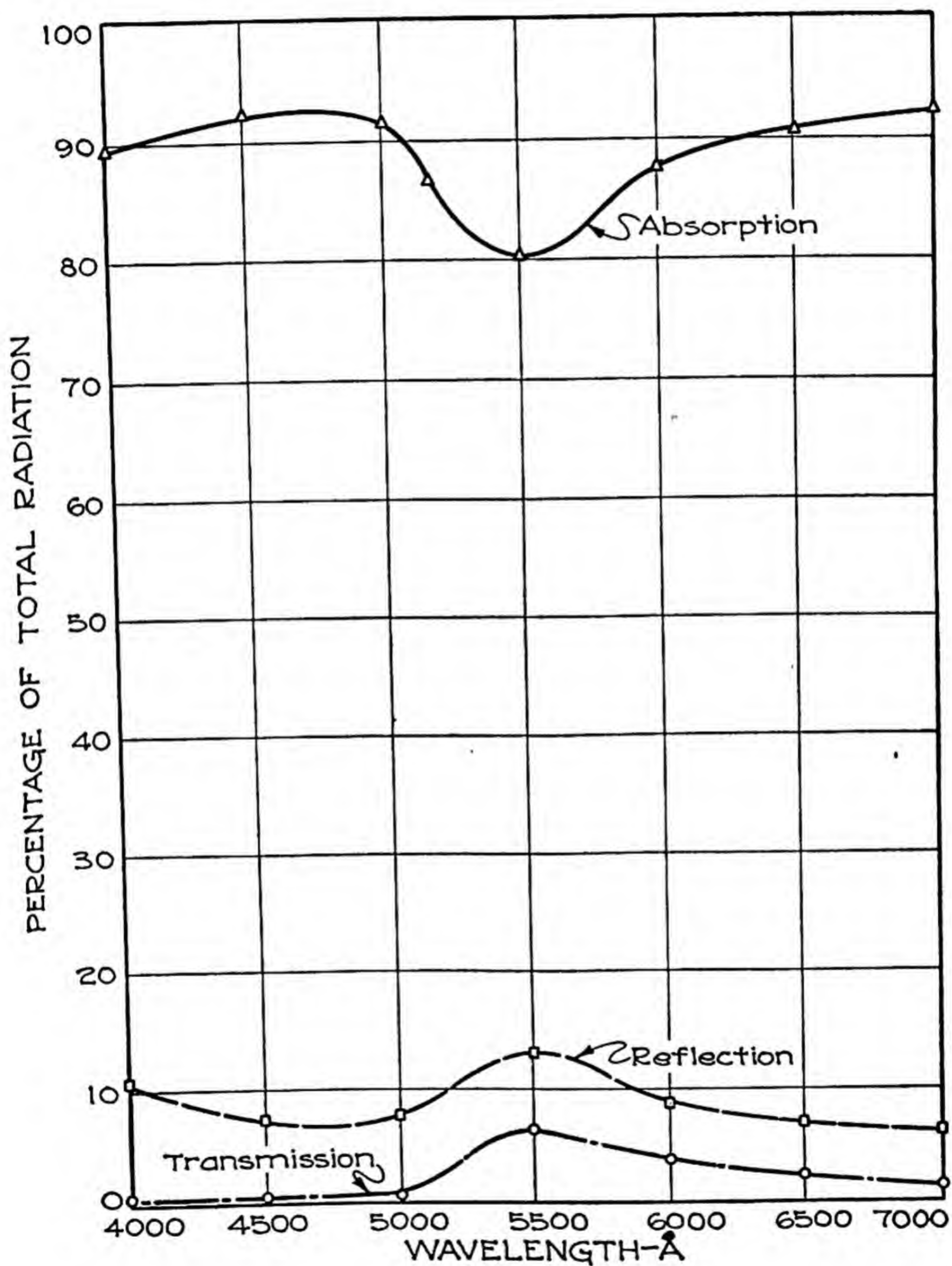


FIG. 1.3—Reflection, transmission and, by difference, absorption of visible radiation by leaves. Average of 90 species.

bodies at temperatures of 10–50°C. indicates that leaves are also especially efficient radiators at these wave lengths, and that they may therefore be cooled by radiation whenever atmospheric conditions are favorable (2).

LIMITING FACTORS IN PHOTOSYNTHESIS

Our photosynthetic equation suggests that the supplies of CO_2 or light, or the accumulation of the end products of photosynthesis

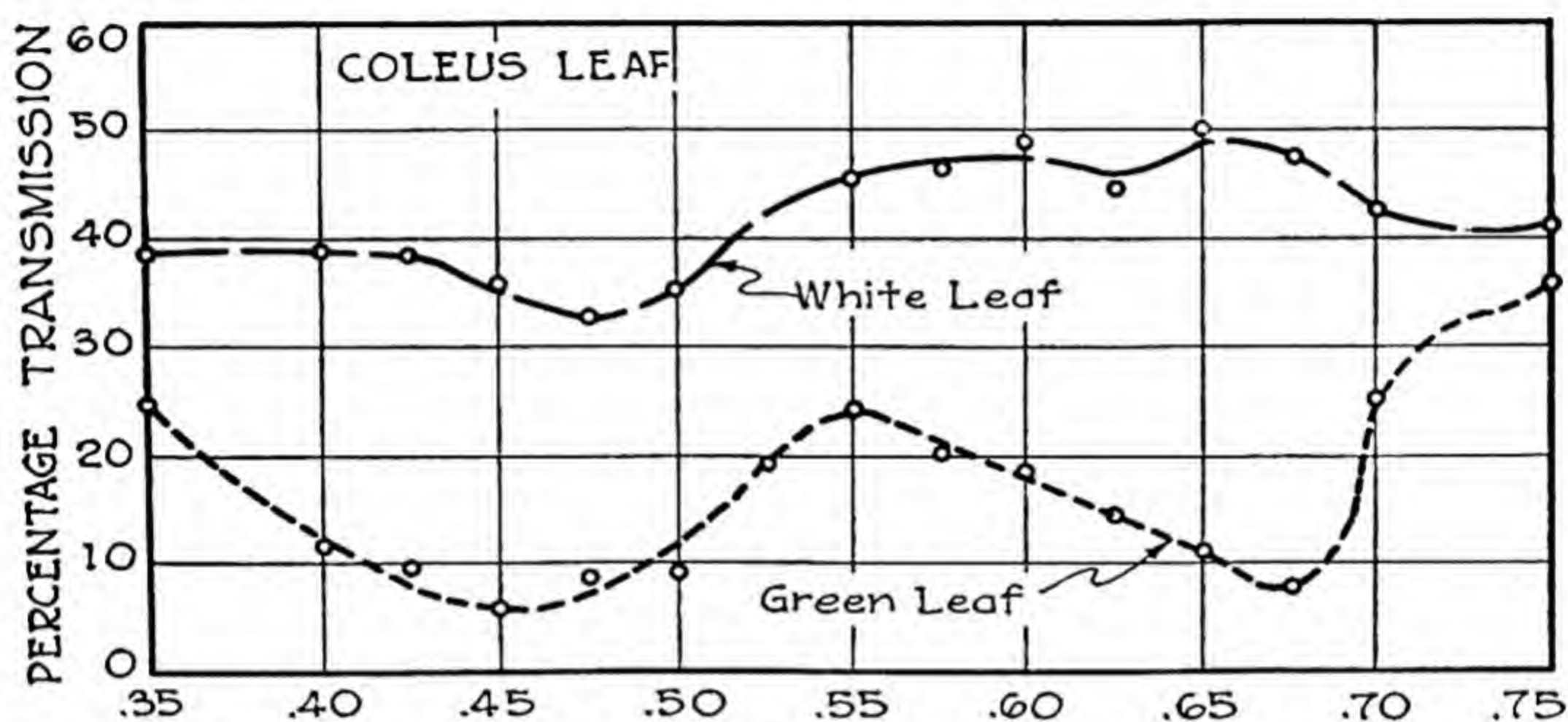


FIG. 1.4—Transmission spectra of the green and nearly white portions of a variegated leaf of *Coleus blumei*.

might become limiting factors for the process. To these we should add temperature, the chlorophyll content and the various unknown enzymic factors of the leaves.

THE CO_2 SUPPLY

Normal air contains about 0.03 per cent CO_2 by volume. In contrast much of the research work on photosynthesis has been done with 4–5 per cent CO_2 ; more than 100 times normal. There is adequate evidence from many sources that higher CO_2 will increase the short time rate of photosynthesis. The data presented by Thomas and Hill in the next chapter are undoubtedly the best available on the effect of increased CO_2 on photosynthesis in growing crops. In short time experiments they obtained linear increases in photosynthesis from CO_2 enrichment of the air to possibly 0.5 per cent. Over a period of two weeks, however, the plants were injured and photosynthesis reduced by daytime enrichment of the air to about 0.3 per cent CO_2 ; ten times normal.

If we consider crop statistics we find that the maximum yields of some common crops are five to twenty times average yields. These maximum crops are produced with essentially the same CO_2 supply as the smaller average crops, the differences being largely ascribable to genetic factors of seed selection and to the supplies of water and mineral nutrients. Also, the largest recorded yields of many plants have been produced in culture solutions under conditions which would tend to reduce the supply of CO_2 below normal.

Our conclusion seems to be that the short time rate of photosynthesis can be increased by CO_2 concentrations up to ten times normal or more, but that these high concentrations are either toxic over longer periods (1) or that other factors of development become limiting (cf. Chap. 17) and the plant is so thrown out of balance as to make a poorer rather than a better growth and dry matter accumulation.

LIGHT INTENSITY

Maximum photosynthesis in a single, normally exposed leaf of corn seems to be reached at about one-fourth of full summer sunlight at Ames (17), 2,500 to 3,000 fc. Both Thomas and Hill (Chap. 2) and Heinicke and Childers (8), however, find that natural masses of leaves, many of them partly to heavily shaded, may respond to greater external light intensities; light saturation for all of the leaves together being reached with 50 per cent of full sunlight or more. Light saturation for *Chlorella*, in contrast, may be reached with 2 or 3 per cent of full sunlight (cf. Fig. 17.1). All of these light saturation values may be raised in short time experiments by increasing the CO_2 supply.

On the lower side, few species of flowering plants survive on the forest floor where the light intensity is less than 2 per cent of full sunlight, about 200 fc. maximum. To survive, plants must produce enough photosynthate during daylight hours to replace 24-hour respiration losses and to make a minimum growth of roots, conducting tissues of the stems and new leaves. The compensation point or light intensity which will just resynthesize the CO_2 produced in respiration varies with the temperature, the species, and its previous history, from a few foot candles to 100 or more. Some mosses and algae grow in caves where the maximum light intensity is 10 fc. and their compensation point must be less than half this value, perhaps 1 or 2 fc. Leaves of field grown corn at temperatures around 30°C . require about 100 fc. to compensate respiration.

Since respiration and growth are reduced more rapidly at lower temperatures than photosynthesis, a lower light intensity is

adequate for plant development in relatively cool weather. This principle is used when the temperature of greenhouses is lowered at night or on dark days.

Technically, some photosynthesis will be carried on at any light intensity above zero, but practically, light of 500 to 1,000 fc. is required for fair rates of photosynthesis in typical leaves. The supplementary lights so widely used in research greenhouses commonly provide 20 to 30 fc. This is more light than necessary for photoperiod responses where 1 to 5 fc. is usually adequate, but not enough to aid significantly in total photosynthesis.

CHLOROPHYLL

An average leaf may be expected to contain about 0.25 per cent chlorophyll *a* plus *b* on a fresh weight basis. Dark green leaves may contain twice as much; yellow-green leaves of some horticultural varieties of ornamentals 75 to 90 per cent less. It is significant that the lighter green forms do not survive in natural competition, but in short time experiments their rate of CO₂ fixation may approach that of normal leaves (7, 20). It seems probable that 0.10 per cent chlorophyll may be adequate for normal growth when no other leaf factors are deficient, particularly if water or mineral supplies are below optimum. On the other hand plants chlorotic because of virus disease, iron, manganese, or other deficiency may show an abnormally low rate of photosynthesis, suggesting that phases of the process other than those directly dependent on chlorophyll are affected.

TEMPERATURE

Many excellent experiments, starting with the work of Blackman and Mathaei (12), have shown a temperature coefficient of $2 +$ for the over-all rate of photosynthesis. Experiments with flashing light (4) have shown, as would be expected, that the temperature response is confined to the enzymatic, dark reactions. The temperature coefficient of 1.0, no increase with temperature, shown by Thomas and Hill (Chap. 2, cf. Table 2.4) for over-all photosynthesis under a wide range of field conditions is therefore somewhat surprising. An examination of the experimental conditions shows that the workers reporting the high temperature coefficients have generally, if not always, used CO₂ supplies higher than those of normal air. Thomas and Hill also used high CO₂ but, unfortunately, made no temperature tests on these runs. Since they were able, however, to increase photosynthesis of crops in the field some five-fold by added CO₂ in short time experiments, the inference seems

to be that 0.03 per cent CO_2 is so limiting that the physical processes of its absorption commonly fix the rate of photosynthesis after light saturation is reached.

The diffusion of CO_2 into leaves is a physical process which would not be expected to show a marked temperature response. It should not be assumed, however, that the leaf is inefficient in absorption. The discussion of Chapter 4 indicates that the difficulty is not to show why so little material diffuses through the stomates, but how interference keeps it to the observed level. The efficiency of the leaf surface and its stomates can be illustrated by experiments with corn in which 100 l. of air was drawn through cellophane envelopes containing about 100 cm^2 of the tip of a corn leaf. The air made irregular contact with the leaf in a stream as much as one cm. thick at points, and passed over the leaf in an average time of less than two seconds. At this speed, with the stomates so nearly closed that no opening could be observed microscopically, 50 to 75 per cent of the CO_2 was removed from the air stream by the absorbing leaf. We must conclude first that the leaf is highly efficient in CO_2 absorption, and second that the absolute amounts of this gas present in the air are seriously limiting for short time rates of photosynthesis.

ACCUMULATION OF END PRODUCTS

If CO_2 concentrations are so seriously limiting, while at the same time plants make such a considerable growth and, from the limited data available, are injured by continued high CO_2 concentrations, it seems probable that the utilization of photosynthate is the final limiting factor concerned in plant development under field conditions. In rather fragmentary data we have not observed a decrease in CO_2 absorption by the leaves of corn plants whose sugar content was increased to several times normal by bagging or removing the ears to reduce translocation. At the same time we (11) and others (9) have observed a marked decrease in the total dry matter accumulated by the bagged plants. Myers' work with *Chlorella* (Chap. 17) indicates that a reduction of growth rate decreases photosynthetic efficiency in this plant also.

It is possible that back reactions may occur when photosynthate accumulates. The suggestion of Franck (Chap. 16) that the enzyme systems of photosynthesis may be inhibited by the narcotic action of the intermediate products of respiration, may be a better explanation however. The accumulation of sugar stimulates respiration and so might produce substances inhibiting photosynthesis whenever the production of sugars runs ahead of their utilization. The

suggestion of the California workers in Chapter 19 that the intermediate products of photosynthesis resemble those of respiration offers a still broader basis for the imbibition of carbon assimilation by the unutilized products of its own reactions.

THE MECHANISM OF PHOTOSYNTHESIS

If we include the discussions of quantum efficiency, 13 of the 22 chapters in this volume are largely or wholly concerned with various phases of the mechanism of photosynthesis. On some points, such as quantum efficiency, there is general agreement among the various workers. On the vital topic of intermediate products, and therefore the general course of the process, we must say simply that more data are needed.

PRELIMINARY DARK REACTIONS

One of the first findings of early tracer research in photosynthesis (cf. Chap. 18) was the dark fixation of CO_2 in a product of moderately high molecular weight and considerable stability. This compound is formed outside the chloroplasts (6), and is therefore not a chlorophyll- CO_2 complex.

Its formation is dependent upon an enzyme system, since it is not formed in dead tissues or in the presence of suitable enzyme inhibitors. Many of the present schemes for the course of the photosynthesis reaction start with this RCOOH compound (cf. Chap. 16 and Fig. 1.5). Possibly the burst of CO_2 evolution at the beginning of illumination, called the Emerson effect (cf. Chap. 10) is caused by the decomposition of excess RCOOH during the transfer to the light reaction forms, or perhaps, as Brown, Fager, and Gaffron suggest in Chapter 20, there is no relation between RCOOH and photosynthesis. If this compound does not serve in photosynthesis, however, it is necessary to postulate a second substance which will serve to bind the CO_2 in reactive form during the reduction steps of the process.

The RCOOH compound is formed outside the chloroplasts (6) and it seems that a compound formed within the plastid would be advantageous. In Willstätter's early hypothesis (20) he assumed that this first compound was between chlorophyll and CO_2 . A number of workers now suggest that there is not a direct chemical union between chlorophyll and CO_2 at any time, but that the CO_2 complex is free to react with any of numerous activated hydrogen donors. The chlorophyll molecule seems to fit best into this role of activatable hydrogen donor.

LIGHT REACTIONS

There is general agreement on two points in the light reactions: chlorophyll must be present, and probably takes an active part; and at least four main stepwise reactions are involved.

The hypothesis that pigments other than chlorophyll may supplement this compound in energy absorption is based on the observation that brown or red algae may show high quantum efficiency in wave length regions where absorption by the extracted pigments is mainly attributable to carotinoids. We have already shown, however, (Fig. 1.2) that absorption by chlorophyll in cells may be very different from that in solution, and we feel that the point of the participation by other pigments is not proven. If future work establishes such participation, we may expect to find that energy absorbed by the carotinoids can be transferred to chlorophyll, but that certain essential steps, e.g., hydrogen transfers, are performed only by the latter molecule.

A schematic presentation of light-reaction steps, taken from Franck (5), is shown in Figure 1.5. Catalyst A should be thought of as an enzyme system responsible for the first fixation, and possibly not identical with the dark fixation system of Ruben, Hassid, and Kamen (cf. Chap. 18, also 19 and 20). Each of the steps in the figure represents the gain in energy possible from the absorption of one quantum ($h\nu$) of light. This energy is presumably absorbed by chlorophyll and is utilized in the transfer of one hydrogen atom to the CO_2 . Very possibly, hydrogenated chlorophyll is the hydrogen donor as well as the energy absorber. With this scheme the CO_2 could react with any of several surrounding chlorophyll molecules, provided they had received the necessary activation by the absorption of radiant energy (cf. Chap. 7). Such an arrangement would fit with many observations on photosynthesis, and simplify the problems of energy transfer.

Another enzyme system, designated as B in the figure, is postulated here to explain phenomena of temperature coefficients, light saturation, etc. If the enzyme B system is limiting, the energy of the activated molecules would be lost as heat until the enzyme could be cleared to stabilize the reaction products. Notice that all of the enzyme reactions are drawn as exothermic, and the energy drop at these points would tend to decrease quantum efficiency.

If four hydrogen molecules are to be transferred to CO_2 in four reactions, and intermediate compounds of the required stability are to be formed, it would seem that the identification of these compounds with C^{14} tracer should be relatively simple. The best data available to October, 1948 are included in chapters 19 to 21. They show astonishing difficulties, and wide divergence in the views of the two main groups working on the problem. Much of the

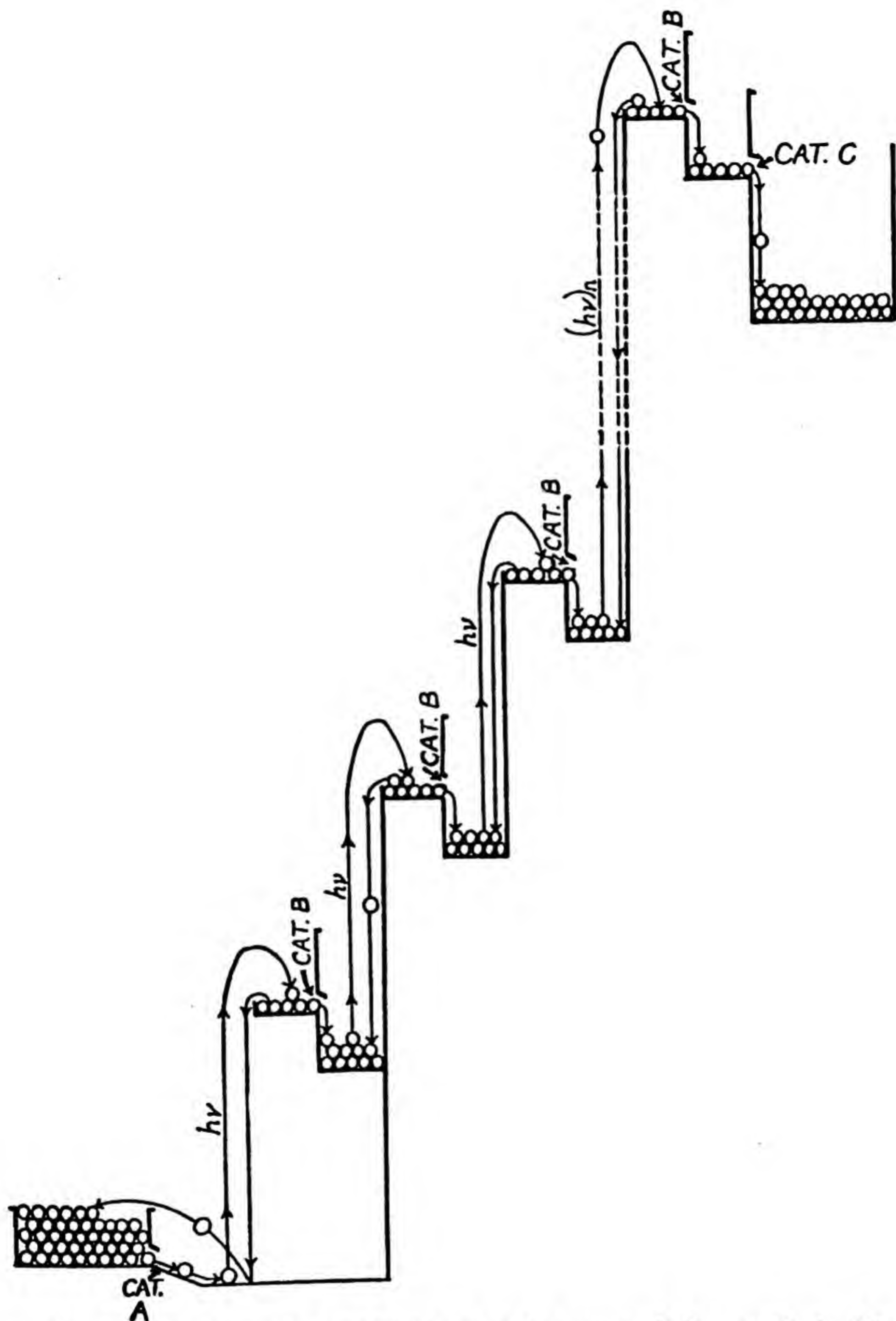


FIG. 1.5—Diagrammatic representation of the course of photosynthesis. After the primary dark fixation by Catalyst A, the CO₂ is reduced by successive steps, each utilizing one quantum of radiant energy ($h\nu$). (From Franck, in Baitsell's *Science in Progress*, Third Series, Yale University Press.)

divergence could probably be eliminated if both groups had worked consistently with carbon fixation in illuminated cells.

Calvin's group reasoned that preillumination would spread out the intermediate steps and give them more time to study the compounds involved. The cycles outlined on page 399 are based on these preillumination results. The data of Table 19.2 show, however, great differences in the distribution of C^{14} in the preilluminated samples, to which $C^{14}O_2$ was added only after transferring to darkness, and the samples receiving the tracer carbon in the light. The acid cycle proposed by this group as the path of photosynthesis permits the ready insertion of CO_2 and H_2 molecules. It is in fact a variation of the Wood-Werkman reaction, which has been demonstrated in bacteria and other organisms, during which non-photosynthetic cells build external supplies of CO_2 into cellular tissues with the energy of respiration. Such a sequence would offer a simple, relatively straightforward solution of the problems of intermediate reactions in photosynthesis. The question to be solved is whether this cycle is photosynthesis or respiratory synthesis. Their own data (Table 19.2) indicate that a different hypothesis might have been developed if direct illumination had been used instead of preillumination.

Gaffron's group seems to have found a specific, remarkably stable, early compound of photosynthesis. It is not used in respiration and is not redistributed in the dark, but goes forward only with continued illumination. Their data suggest a specific compound or compounds unrelated to ordinary plant metabolism. Perhaps the most remarkable aspect of their "B" fraction, however, is its resistance to classification and identification.

We feel reasonably sure of our postulation of the reduction of CO_2 by hydrogen. We do not know whether chlorophyll is directly concerned in hydrogen transfer, although we feel that it probably is. The biggest unsolved problem is that of intermediate compounds. We can assume, however, that the Chicago group will be able to isolate and characterize their "B" substance (fraction?), and that the California workers will continue their important chemical work, shifting, however, to tests of products formed in light. Such efforts should solve this problem in the near future.

The attention of lay plant physiologists is directed in the meantime to the paucity of information on photosynthesis in higher plants under approximately normal conditions of plant development, CO_2 supply, etc. In spite of the concentration of groups of specialists on the problems of photosynthesis, there is still much work to be done with simple apparatus measuring the effects of variety, disease, growth conditions, and environment on photosynthesis in plants.

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Photosynthesis Under Field Conditions

MOYER D. THOMAS AND GEORGE R. HILL

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THE PROCESS of photosynthesis involves many simultaneous reactions. In their study, much of the experimental work has been done on the simplest systems, particularly on the one-celled plants in the Warburg apparatus. This has permitted rigid control of the experimental conditions. Other simple aquatic plants have been similarly studied, but investigations of the higher plants usually have been confined to observations on the leaves, either attached or abscised.

Only a limited number of investigations have been carried out on complete systems of higher plants growing under natural conditions, though this would be desirable for its own sake and also to observe the applicability to the higher plants of information obtained from the study of the simpler systems. Of course, since most of the dry weight of the plant is derived from the products of photosynthesis, yield data may be regarded as measuring this quantity, but yield data alone supply no information about the relative amounts of respiration and photosynthesis, or the detailed effect of environmental factors. In addition, it is usually impracticable to include the root system with the yield data.

This chapter is devoted principally to work on the photosynthesis of crop plants carried out in the Department of Agricultural Research, American Smelting and Refining Company. Parallel studies of Heinicke and his co-workers on trees are considered briefly.

Photosynthetic data are obtained by measuring the gas exchange of the plants growing on six-foot-square plots. Practically, this means measuring the CO_2 exchange, since no method of sufficient rapidity and precision is available for determining slight changes in the O_2 content of the air. It has been shown that the photosynthetic and respiratory quotients are approximately unity. Accordingly,

CO₂ exchange should be a reliable measure of these processes. Since CO₂ is present in the air to the extent of only 0.03 per cent, changes in this constituent due to respiration and photosynthesis may be relatively large, and it is possible to measure them with a high degree of precision.

The use of plots instead of single plants or single leaves greatly reduces the variability of the measurements. Simultaneous measurements of the assimilation on two similar plots have shown hourly deviations of only 1 to 5 per cent, whereas Verduin and Loomis (22) encountered average variations of 25 per cent and maximum variations of 100 per cent in the assimilation rates of comparable corn leaves under the same external conditions. Heinicke and Hoffman (7) found similar variations of as much as 500 per cent. However, Hoover, Johnston, and Brackett (8) were able to attain remarkably close reproducibility using a single wheat plant in a controlled environment.

APPARATUS

One of the essential requirements for this work has been the development of an automatic analyzer which would measure continuously the CO₂ concentration. Such automatic equipment was first described in 1933 (15, 18).

A diagram of the apparatus is shown in Figure 2.1, which illustrates two absorbers and a valve system for controlling the flow of liquid and air. The absorbers are capable of scrubbing out practically all the CO₂ from a stream of air passing at about 300 ml./min. The absorbing solution is 0.005 N sodium hydroxide, containing 1 per cent butyl alcohol. A sintered glass disk breaks up the air stream into very fine bubbles, and the alcohol causes foaming and thus prolongs the period of contact between the bubbles and the liquid. The progress of the absorption is determined by measuring the electrical conductivity of the solution as it changes from hydroxide to carbonate, the latter having about one-half the electrical conductance of the former.

Absorption is aided by maintaining the temperature in the water bath surrounding the absorbers at 35–40° C. Evidently, one of the limiting factors in the absorption of CO₂ is the rate of hydration of the molecule when it is dissolved in water. This is a slow reaction which is favored by an increase in temperature, and the increased rate of hydration more than offsets the decreased solubility of the gas at the higher temperature.

Figure 2.1 also illustrates an excellent method of dispensing pairs of accurately-measured air samples. A pair of flat, steel boxes, 15 x 20 x 2 cm., connected by a U-tube, contains enough mercury to

fill one box and the U-tube and leave 600 ml. of free space in the other. When suction is applied to the equalizing bottle, which has a bleeder valve to prevent the pressure in the bottle falling more than 5 cm. Hg below atmospheric pressure, the mercury in the box, under the control of valves *D*, *E*, *I*, *J*, *K*, and *L*, flows alternately from one box to the other, thereby dispensing an air sample before it and drawing in a fresh sample behind it. The rate of flow of the gas from the meter through the absorbers is adjusted by two needle

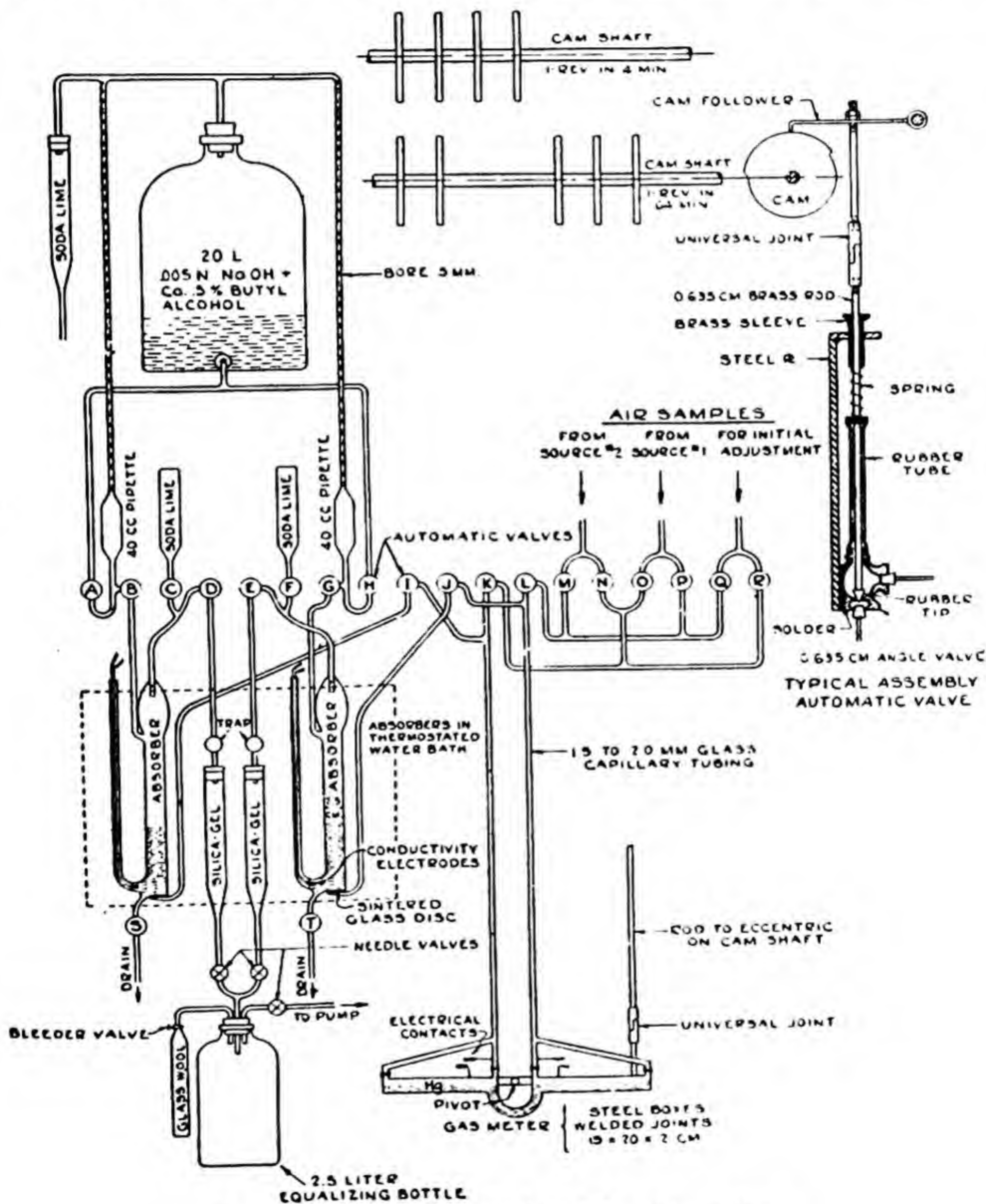


FIG. 2.1—Diagram of the CO₂ autometer.

valves on the suction system, so that the entire gas sample is withdrawn from the meter in a few seconds less than the two-minute period allotted for the operation. Mercury is then drawn up into the glass capillary tubing above the meter a distance of about 5 cm. and no more gas flows until the end of the two-minute period. Then the valves change and the direction of flow of the mercury is reversed. While one absorber is operating, the other is quiescent.

The cycle consists of sixteen two-minute periods, giving eight samples to each absorber, which retains the same absorbing solution throughout the cycle. At the end of the cycle, the absorbers are recharged with liquid and the source of the samples is reversed. By averaging two consecutive cycles, any slight machine differences may be cancelled out. In an extensive experiment, the standard error of the difference in the registration of two absorbers sampling from the same source of air was 0.25 per cent. In Tables 2.1 and 2.2, comparisons of net assimilation and yield data indicate satisfactory operation of the autometers over long periods of time.

CO₂ RECORDER CHART

The type of record obtained is illustrated in Figure 2.2 by a section of the recorder chart. This chart gives the intake and outlet concentrations of an air stream passing through a plant chamber containing alfalfa. The conductance of each absorber is measured after each absorption period during the time the absorber is quiescent, since it is impossible to measure the conductance when the gas is bubbling through the solution.

The method of averaging successive cycles is indicated, and the differences between intake and outlet concentrations, due to photosynthesis before sunset and to respiration after sunset, are shown. The track at the right margin, which is made by a contact in the glass tubes above the gas meter boxes, proves that a full air sample was dispensed for each absorption. Just before sunset the air volume passing through the plant chamber is reduced to one-third of the daytime volume. The smaller differences due to respiration can then be measured accurately.

It may be noted that the concentration of CO₂ in the air rose from 292 ppm. in the afternoon to 330 ppm. in the evening. Concentrations as high as 400–500 ppm. are frequently observed during the night. A rise in the concentration of CO₂ in the air occurs regularly in the evening, and the reverse effect occurs in the morning. This is doubtless caused by depletion of CO₂ in the air close to the ground due to photosynthesis in the daytime, and by enrichment due to respiration at night. The concentration of CO₂ near the ground is generally raised higher above its average

value at night by respiration than it is reduced below the average in the daytime by photosynthesis, even though the latter rate is considerably greater than the former. Temperature gradients in the air near the ground during the daytime favor dispersion of the gas and at night favor accumulation.

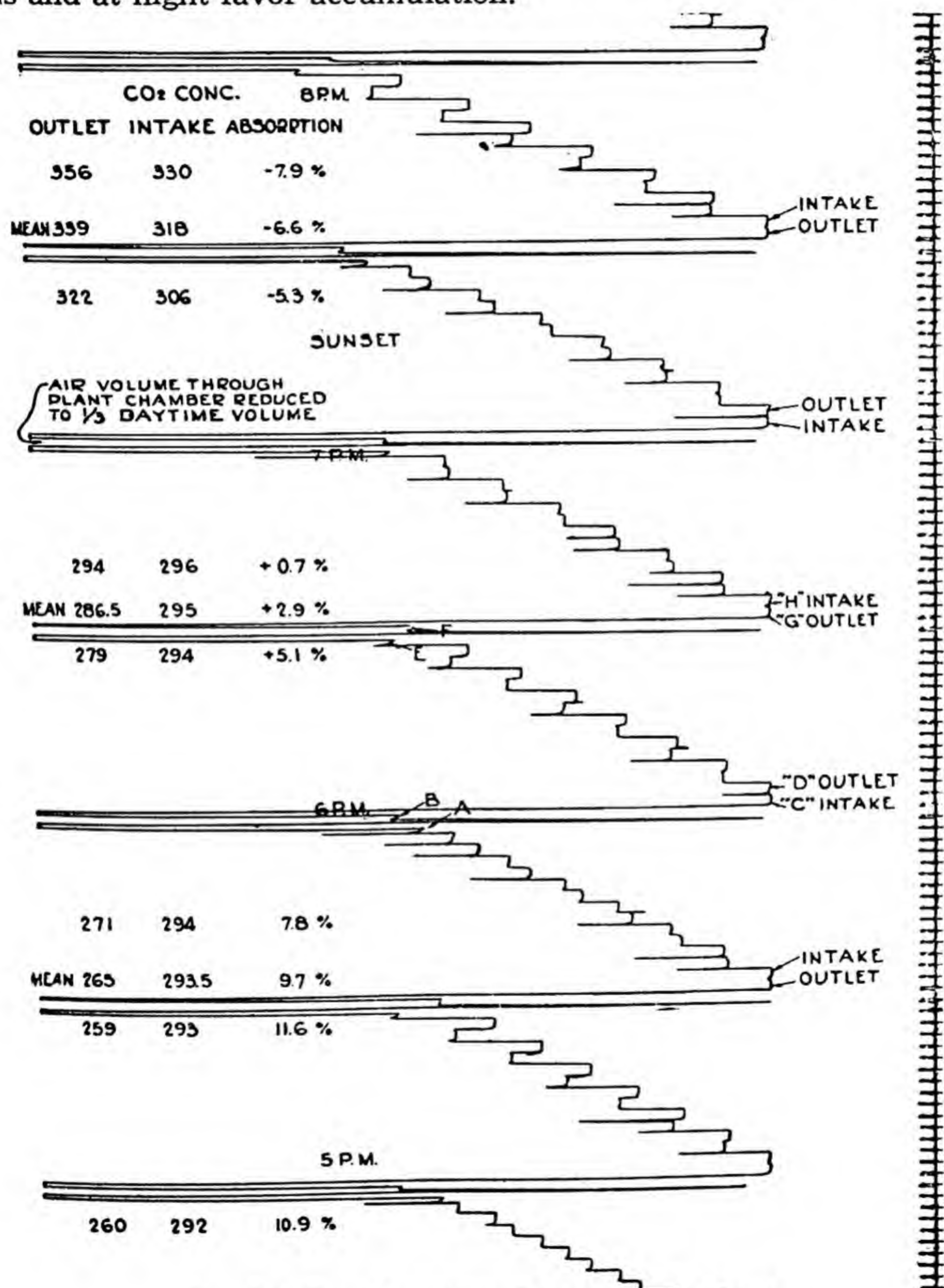


FIG. 2.2—Sample section of the recorder chart.

It will be noted in Figure 2.2 that the difference in concentration between intake and outlet during photosynthesis is small. In practice the difference is seldom allowed to exceed 15 per cent, in order to avoid the effect of an atmosphere depleted in carbon dioxide on the photosynthetic process.

A photograph of a CO₂ autometer, having four absorbers instead of two, which may be used for comparing two plots simultaneously is shown in Figure 2.5. This machine has additional valves on the air line so that a four-way reversal of the source of samples is made on four successive cycles.

Considerable simplification in the construction of this equipment may be accomplished by the use of multiple-port stopcock valves (20) in place of the poppet valves illustrated in Figure 2.1. In practice, it has been found desirable to increase the absorption period from 2 to 2½ minutes, and the double cycle from 64 to 80 minutes. These longer periods generally give adequate detail for the exchange curves throughout the day and night. If more detail is needed, the 2½ minute absorption periods can, of course, be calculated individually.

OTHER CO₂ ANALYZERS

A new analyzer has recently been developed which gives a nearly instantaneous registration of the CO₂ concentration and an integrated value after any desired period of accumulation as well. The machine is shown diagrammatically in Figure 2.3. The absorber consists of 20 to 25 feet of 5 mm. (o.d.) glass tubing coiled in a spiral about 5 cm. in diameter. Liquid and gas are fed into this coil at a constant rate. After emerging from the coil, where complete absorption of the CO₂ occurs, the liquid and gas are separated, and the conductivity of the solution is measured as it flows through a plastic cell containing platinum electrodes. The liquid is collected in an accumulation vessel and is well mixed by the gas stream. Its conductance is measured periodically, or after a definite amount has accumulated, before it is discharged from the accumulating cell.

The success of this system depends on dispensing the liquid and gas at accurately controlled rates. The gas is dispensed at 300 ml./min. by a Zenith gear pump (23) provided with a continuously-operating oiling system. These precision pumps, when properly lubricated, have been found to deliver a very uniform volume of air, even against a head of several centimeters of mercury. The dispensing of the absorbing solution with this type of pump has not been satisfactory because it was not practicable to provide continuous lubrication. However, a satisfactory liquid stream can be obtained using a simple pump made by squeezing a piece of heavy-wall rubber tubing between glass check valves. The most

satisfactory operation has been obtained by squeezing the rubber at three-second intervals. This apparatus should be particularly useful for recording short-time fluctuations in the CO_2 exchange. It has a sensitivity of about 1 ppm. CO_2 and the registration represents a running average concentration covering 1-2 minutes of sampling. It offers no advantages over the older system for protracted measurements of the exchange.

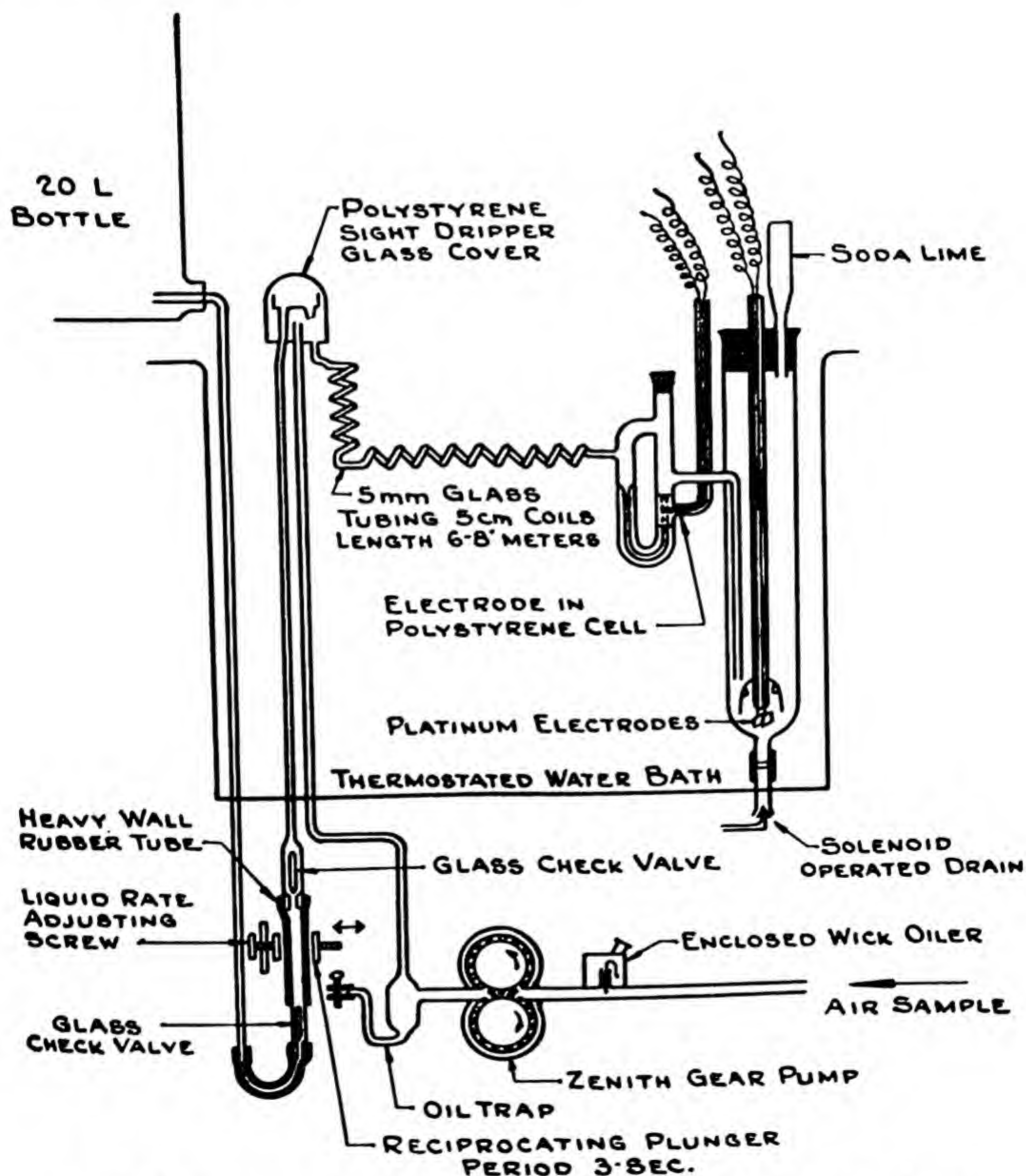


FIG. 2.3—Diagram of an automatic "instantaneous" type CO_2 analyzer.

A third type of CO_2 analyzer has been extensively employed for root respiration measurements. This machine is partly automatic in that it delivers a measured volume of air sample to the absorber. The absorbing liquid, however, is measured by means of a hand-operated, automatic pipette, and the solution is drained from the absorber and titrated whenever desired. Figure 2.4 illustrates diagrammatically the essential features of this apparatus. It is

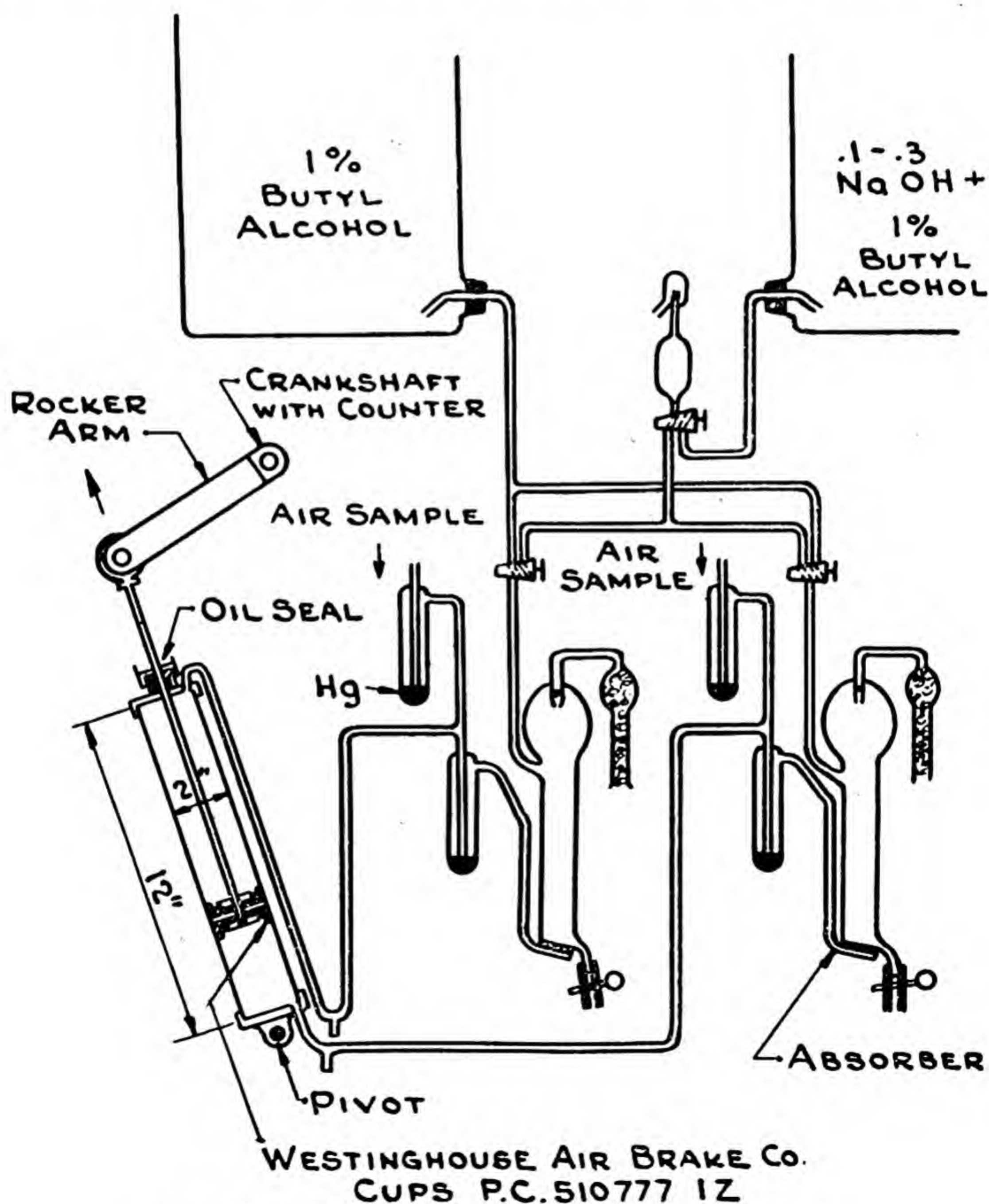


FIG. 2.4—Diagram of a partly-automatic CO_2 analyzer.

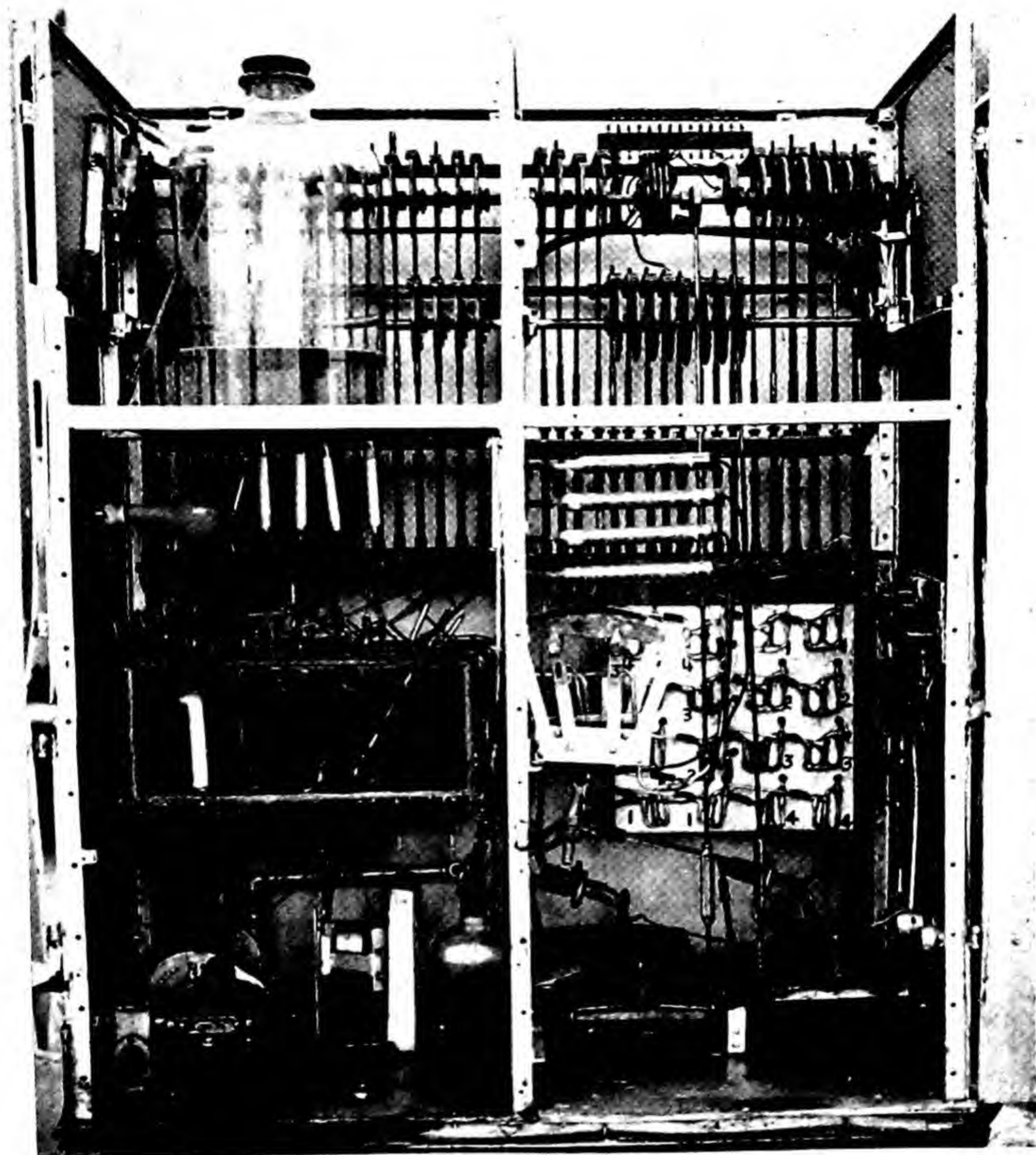


FIG. 2.5—The CO₂ autometer for sampling air from four sources.

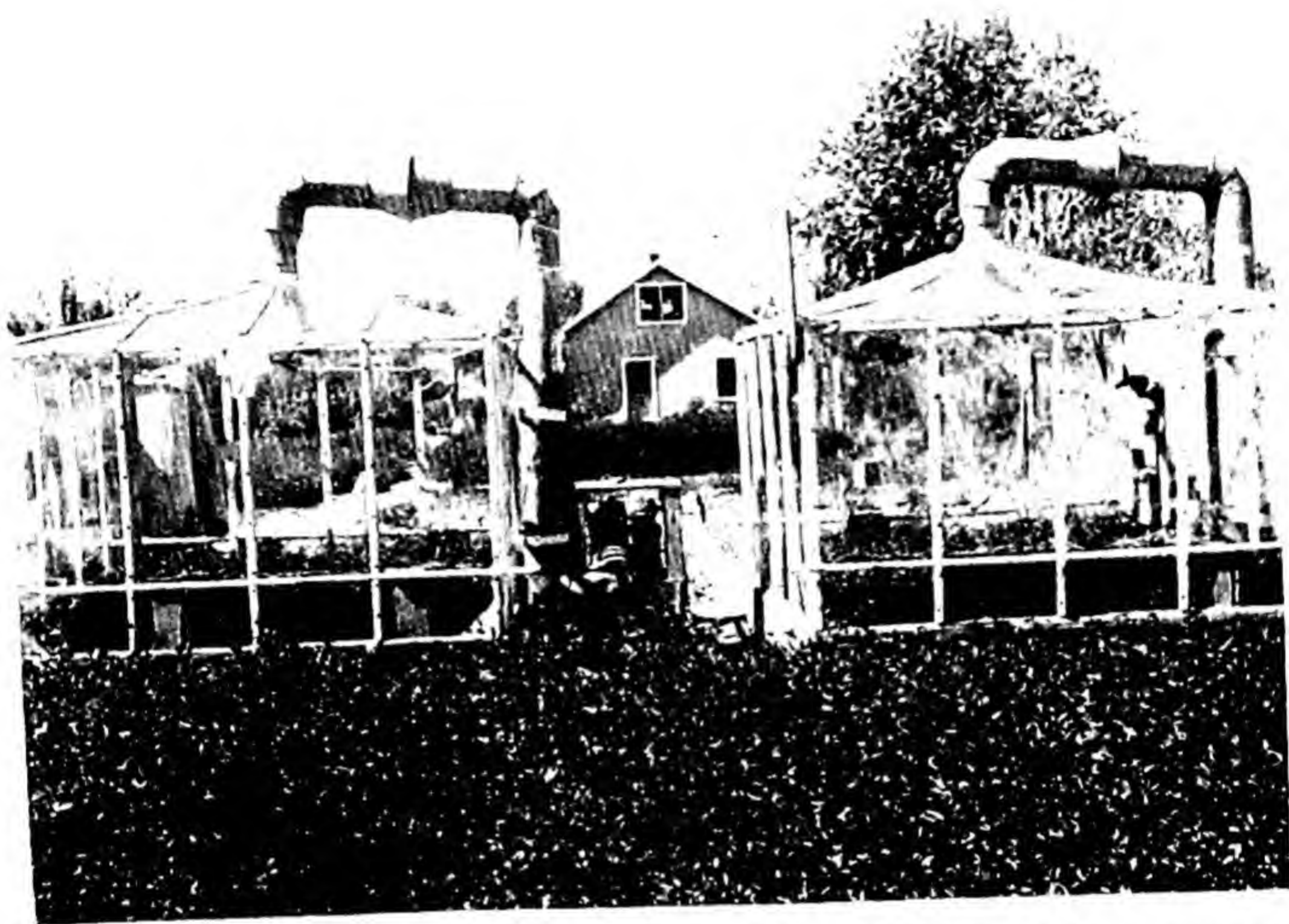


FIG. 2.6—Portable, celluloid-covered cabinets suitable for field measurement of the CO_2 exchange.



FIG. 2.7—View of the sand culture equipment.

provided with a piston-type pump. The plunger of this pump has two plastic cups which are resistant to oil and water and which have operated without leakage for nearly six years. This pump makes a full stroke in each direction in 2.5 minutes. When it moves in one direction, it draws an air sample behind it and forces a previously measured air sample out ahead of it through the absorber. Control of the air samples is accomplished by four simple mercury check valves which cut off the connection to the absorber when the pump is applying suction, and cut off the connection to the source of sample when the pump is applying pressure. One pump, therefore, takes care of two air samples and two absorbers.

In one assembly of this apparatus, four pumps and eight absorbers are employed. Automatic pipettes of two sizes are also provided, and the solution of NaOH usually used has a strength of 0.1–0.3 N. The absorbing solutions can be replaced with fresh absorbent without stopping the machine. Time of sampling may be varied from a few minutes to 24 hours, the actual volume of gas being indicated by a counter on the crankshaft which operates the pumps. It is quite practicable to drain and replace the eight solutions during 7½ minutes, and titrate them in about 10 minutes, so that eight analyses can be completed in each 20-minute period. Single pairs of analyses can be done each five minutes. The carbonate is precipitated with an excess of barium chloride and the solution is titrated with standard HCl to the thymol blue end point.

In the analyzer developed by Heinicke and Hoffman (7), the air sample is drawn by a pump through a wet-test gas meter at a rate of about 700 ml. per minute; then the CO₂ is absorbed in a large tube containing a fritted glass disk. The absorbing solution is drained out periodically and titrated. Waugh (23) made the method applicable to small air samples by substituting electrolytic conductivity for titration.

PLANT CHAMBERS

Two types of plant chambers have been used in this work (17, 18). A portable, celluloid-covered, iron framework, six feet square and five to six feet high, as illustrated in Figure 2.6, has been particularly useful for the study of crops growing in the field. This cabinet is provided with a blower that sends as much as 500 cubic feet of air per minute into the top of the chamber and allows it to emerge from a single exit pipe near the ground level. The chamber is essentially gas-tight. When the air volume entering and leaving the chamber is measured by means of anemometers placed in the pipes, and the intake and outlet CO₂ concentrations of these air streams determined, the respiration and photosynthesis of the plants in the chamber can be calculated.

In addition to this apparatus, sixteen units of sand culture equipment, described in detail elsewhere (17), such as that illustrated by a general view in Figure 2.7, and further illustrated diagrammatically in Figure 2.8, have been used. This equipment provides a measured volume of washed and filtered air to the plant chambers. Alfalfa, grain, sugar beets, and tomatoes have been grown, and

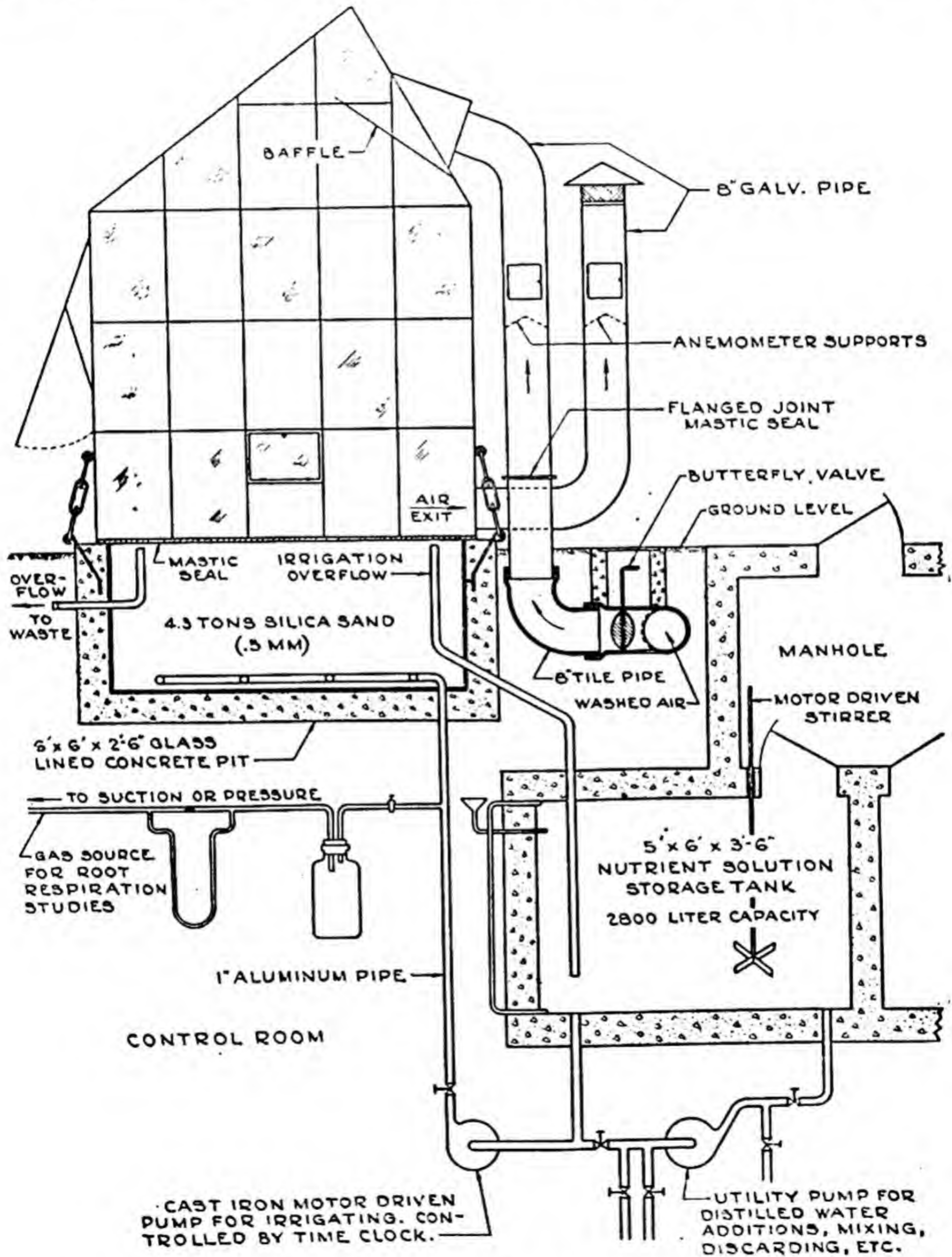


FIG. 2.8—Diagram of one unit of the sand culture equipment.

data on photosynthesis, top respiration and root respiration have been obtained on each of these crops. These observations, together with an extensive series of similar measurements on plants growing in the field, have revealed a definite pattern for the CO_2 exchange curves as they are influenced by external factors such as light, temperature, mineral nutrients, drouth, and other special treatments.

Heinicke and Childers (6) have employed a glass cabinet large enough to enclose a young apple tree of bearing age. This structure has a wooden floor and is provided inside with mixing fans and a refrigeration unit for control of the environment. The measured air stream enters around the trunk of the tree and is drawn off at the top of the cabinet.

THE DIURNAL ASSIMILATION

CLOUDLESS DAYS

Figure 2.9 illustrates some assimilation curves on cloudless days. The lower curve to the left of the chart represents alfalfa which was just starting to grow vigorously, the plants being 6 to 8 inches high. Part of the vegetation was shaded early in the morning and late in the evening by the base of the cabinet so that the beginning of assimilation was delayed in the morning and its completion hastened in the afternoon. The upper curve represents a full-grown plot at about the same time, with the plants about four feet tall. The center curve represents the latter plot when the plants were 18 inches high. It is interesting to note that the midday assimilation level was only twice as great in the plot with plants 48 inches tall and having 3.6 times the weight of leaves of plants 6 to 8 inches tall, due at least in part to a larger proportion of shaded leaves under the fully-illuminated surface leaves of the larger plants.

The chart on the right illustrates three comparable plots at approximately monthly intervals. The chart shows the effect of length of day, but indicates about the same maximum assimilation level, regardless of the fact that the maximum angle of incidence of the sun on September 29 was 47° as compared with 66.5° on August 4. Indeed, the apparent assimilation level on September 29 was somewhat higher than on earlier dates, which may be a reflection of the lower rate of daytime respiration due to lower temperature.

On August 4 and September 29, the respective temperatures at night were 17° and 9° C.; at noon, 27° and 21° . Calculating day respiration from night respiration, using $Q_{10} = 2.0$, the estimated photosynthesis at noon on August 4 was 28.0 gm. per period, and

on September 29, 27.7 gm. Total dry matter at harvest on August 6 weighed 2,330 gm., and the leaves, 650 gm. On October 2, these values were 1,725 and 670 gm., respectively. Since the amounts of leaves and photosynthesis on the two plots were so nearly the same, it is likely that neither light nor temperature were important variables affecting midday photosynthesis on these two days.

Many investigators—for example Harder (5) and Kursanov (10)—have described assimilation curves under uniform light having a decided midday minimum separating two prominent

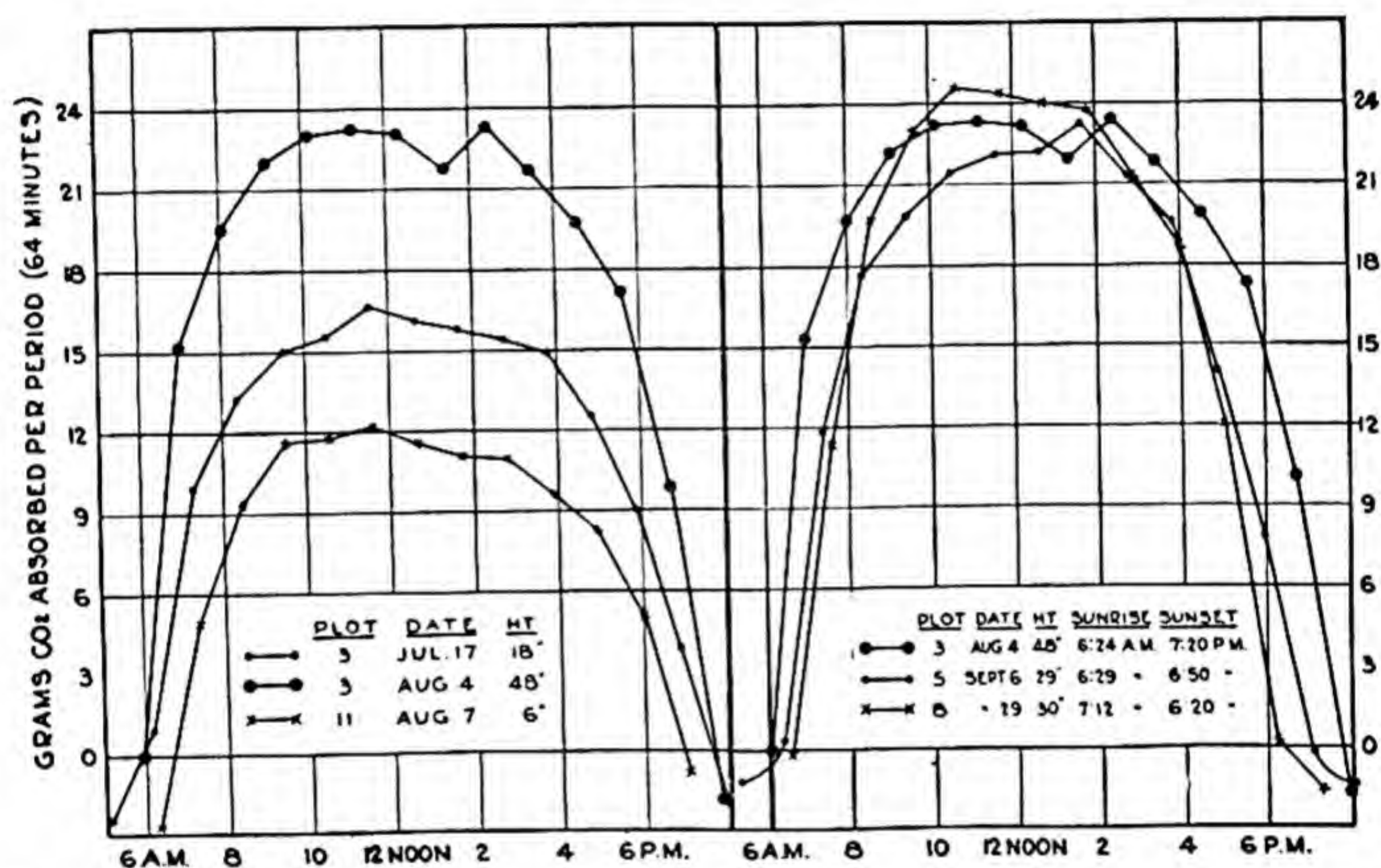


FIG. 2.9—Diurnal assimilation of CO_2 by alfalfa on cloudless days at three stages of growth in midsummer (left), and at monthly intervals (right).

maxima. The minimum has been ascribed to an accumulation of carbohydrates in the leaves, with or without stomatal closure. Recently, Franck (3) has suggested that the chloroplasts may become narcotized by organic acids produced from organic peroxides which normally would be dismuted under the influence of "Cat. C" to alcohols and oxygen. If Cat. C is inactivated, or the concentration of carbohydrates in the leaves is too high, photosynthesis may thus be seriously curtailed.

This so-called "midday depression of photosynthesis" on cloudless days has not been observed with certainty in these studies, though at times the top of the assimilation curve is flattened, particularly with sugar beets. Possibly the slight dip in the August 4 curve in Figure 2.9 is midday depression. The duplicate plot behaved in exactly the same way (19). Evidently the plants in these experi-

ments were able to dispose of the products of assimilation throughout the day so that photosynthesis could proceed without interruption. Also, there was no serious midday stomatal closure.

VARIABLE LIGHT

The influence of cloudy skies is illustrated in Figure 2.10, in which the assimilation curves on one cloudless day and two cloudy days are shown above the corresponding light intensity curves, as given by a recording ten-junction pyrliometer placed

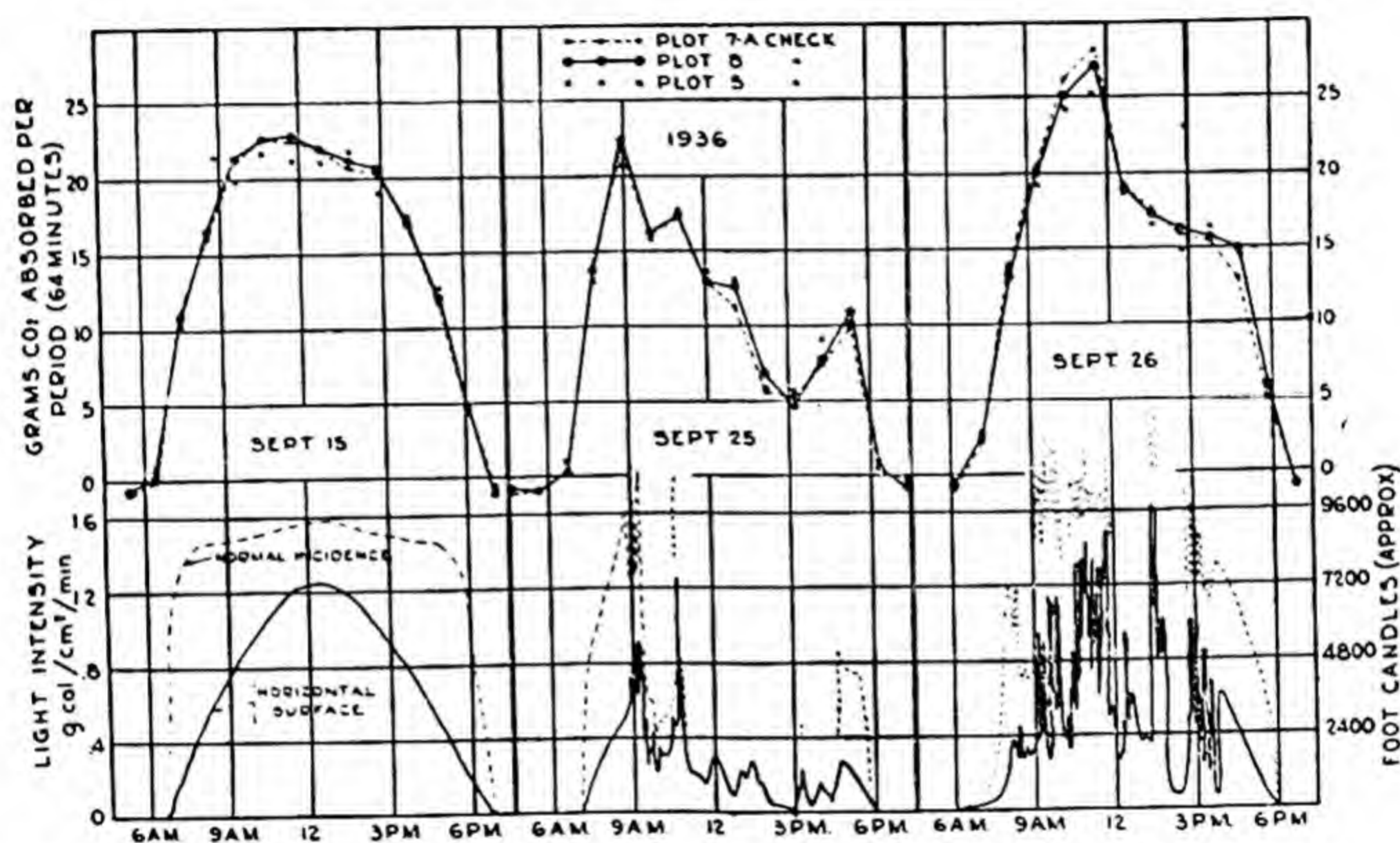


FIG. 2.10—Diurnal assimilation of CO_2 by three similar plots of alfalfa measured simultaneously on a cloudless day and on two cloudy days. The light intensity curves are placed below the assimilation curves.

horizontally. The values of the light intensity at normal incidence have also been calculated from the values on the horizontal surface, and are shown as dotted lines on the light charts.

September 15 was a cloudless day, and the assimilation curves for three similar plots of alfalfa are essentially regular. Their shape is intermediate between the shapes of the light curves at normal incidence and on the horizontal surface. September 25 and 26 were partly cloudy. The peaks of photosynthesis correspond with the peaks of light intensity. Of course, the method of averaging 64 minutes of record in calculating the points on the assimilation curves tends to smooth out any irregularities due to frequent changes in the intensity of the light. For example, on September 26, a different grouping of the data would have shown an assimi-

tion level equivalent to 10 gm. between 1:30 and 2:40 P.M., and 20 gm. between 2:40 and 3:10 P.M. The strong sunlight between 1:20 and 1:30 P.M. can be clearly distinguished in the detailed recording of the CO_2 exchange.

A striking relationship is illustrated on September 26. The photosynthetic maximum was appreciably higher than normal just before noon. This appears to have been associated with the large fluctuations in light intensity due to frequent obscuring of the sun with clouds. It has frequently been noted that higher-than-normal apparent assimilation levels are reached when the sun is intermittently obscured by clouds for a few seconds to 1 or 2 minutes. This increase is probably due, in part, to a lower leaf temperature with attendant lower respiration. It may also be due in part to the orientation of the chloroplasts and to the removal of sugars during the darkened interval. This case is not comparable to that described by Garner and Allard (4), who found minimum growth when plants were grown with alternate light and dark periods of one minute, because in this present case there was enough light for considerable photosynthesis during the cloudy period.

The effect of intermittent cloudiness has also been observed in an experiment in which the atmosphere in a plant chamber was not renewed, thus allowing the concentration of CO_2 to fall until respiration and photosynthesis balanced each other, giving an apparent equilibrium concentration of CO_2 . Figure 2.11 gives the detailed fluctuations of the five-minute CO_2 recordings, representing apparent equilibrium between photosynthesis and respiration, and also the corresponding light intensity values. When light intensity was high, the rate of photosynthesis was increased and the carbon dioxide concentration was decreased. The reverse occurred when clouds reduced the light intensity. The only obvious exceptions occurred at 11:50 A.M. and 2:30 P.M., and are probably analytical errors. Evidently, a definite balance was established in this plot between respiration and photosynthesis, which was shifted rapidly as the light intensity changed.

DROUTH

The effect of slight drouth on the assimilation curves is illustrated in Figure 2.12, which gives the apparent assimilation and respiration of two similar alfalfa plots on two consecutive days. Plot 7A had more impervious soil than the other, and an irrigation on September 22 failed to penetrate uniformly, leaving one side drier than the rest. Assimilation on this plot was reduced during the hot part of the day on September 24, at which time some of the leaves showed signs of wilting. The plots were heavily irrigated on

the afternoon of September 24. Subsequently the assimilation curves came into close agreement again. The respiration curves remained nearly coincident. Verduin and Loomis (22) found that wilted corn leaves absorbed on the average only 37 per cent as much CO_2 as turgid leaves.

SULFUR DIOXIDE FUMIGATION

The assimilation curves have been used extensively to show the immediate effect of gas treatments, such as known concentrations

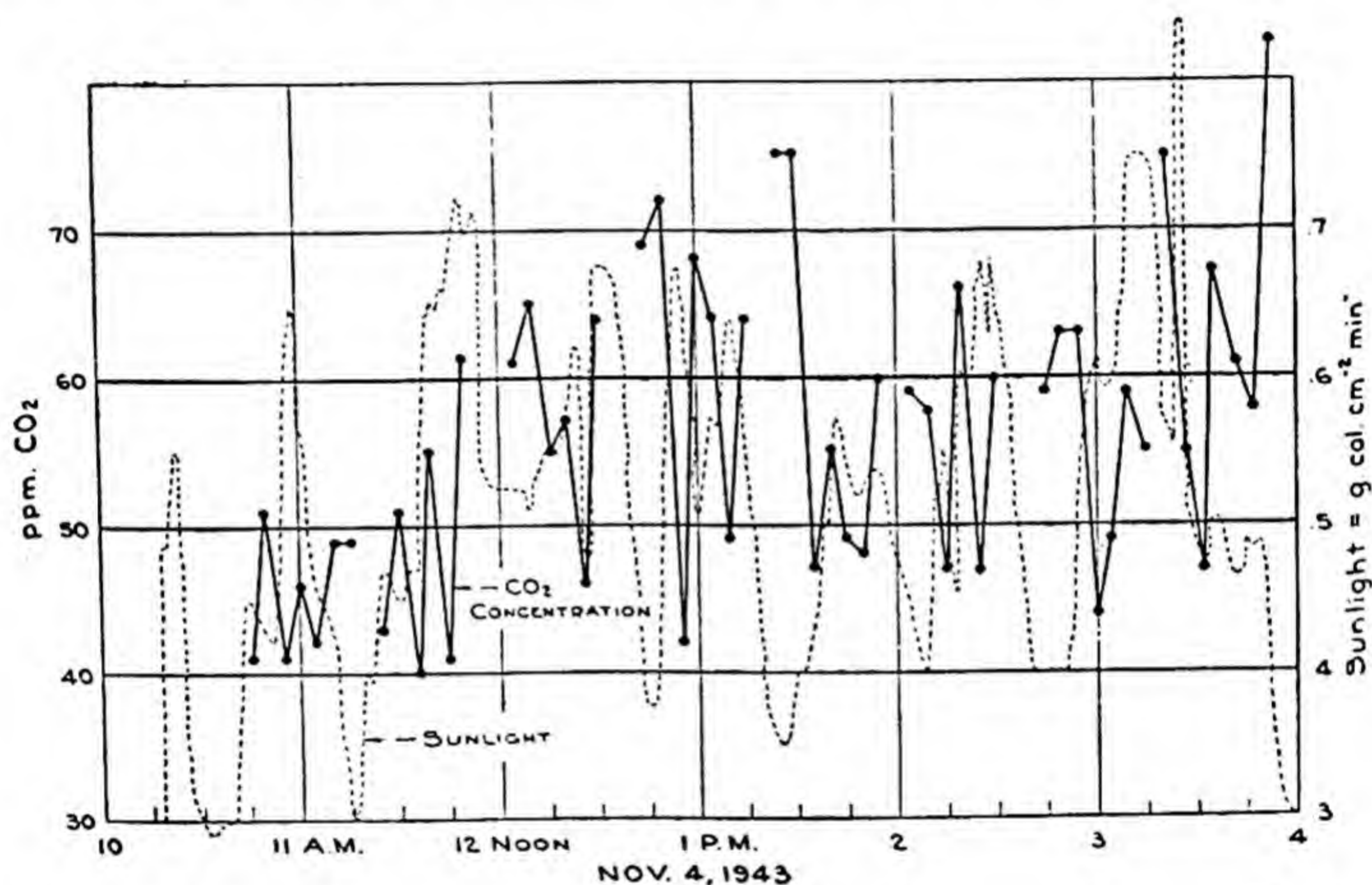


FIG. 2.11—Short period (5-minute) variations of the apparent equilibrium between photosynthesis and respiration with variable light intensity on a partly cloudy day, of sugar beets.

of sulfur dioxide. Katz, Ledingham, and Harris (9) also have carried out a large number of fumigation experiments, using photosynthesis and respiration to measure the effects. Their results agree closely with our own, and the three experiments that follow are typical of both investigations.

In one experiment a short fumigation with a high concentration of sulfur dioxide was applied. This destroyed 95 per cent of the leaf area of the plot. Only a few of the lower leaves in the center of the plot were uninjured. Immediately after the fumigation the plants ceased assimilating and actually commenced to evolve CO_2 as if they were in the dark. On the next two days assimilation and

respiration practically balanced each other, but for a few hours each day there was a small amount of net assimilation. Thereafter, with the gradual appearance of new leaves, the plot increased in ability to assimilate, until on the ninth day the activity was about 65 per cent of the corresponding check plot. Allowing for the fact that before the fumigation the treated plot was 15 per cent less active than the check, the recovery on this date was about 75 per cent.

Figure 2.13 gives the results of an experiment in which one of a pair of similar plots was treated on five successive days with 0.43–

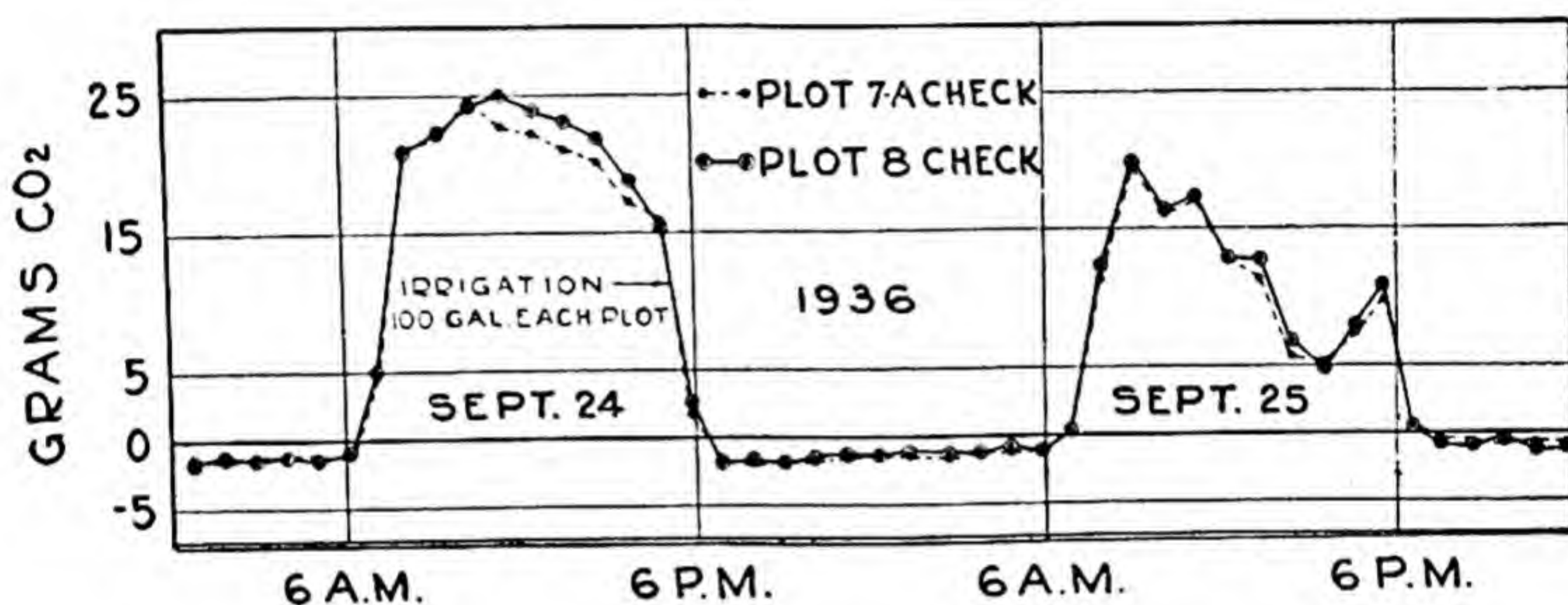


FIG. 2.12—The effect of slight drouth on the assimilation of alfalfa.

0.46 ppm. sulfur dioxide for four hours. These treatments and five other similar fumigations applied earlier on this crop caused only traces of leaf injury. The treatments in every case reduced the photosynthetic activity of the treated plot during the time the fumigation was in progress, but within two to three hours after the fumigation was discontinued the assimilation was normal again. The average rate of assimilation during the progress of these five fumigations was 86 per cent of the check, and 93 per cent during the first five fumigations. The total loss of assimilation due to the ten treatments was estimated to be only 1 per cent, because assimilation on the treated plot increased a little as compared with the check subsequent to the fumigations. The photosynthetic technique, therefore, serves to detect fumigation effects which otherwise would be completely obscured in the yield data.

An experiment in which a concentration of 0.1 to 0.2 ppm. sulfur dioxide was applied continuously for 45 days shows that this treatment had very little effect on the assimilation of CO_2 . The fumigated plot appeared to improve a little with respect to the check plot during the first two-thirds of the experiment. Later,

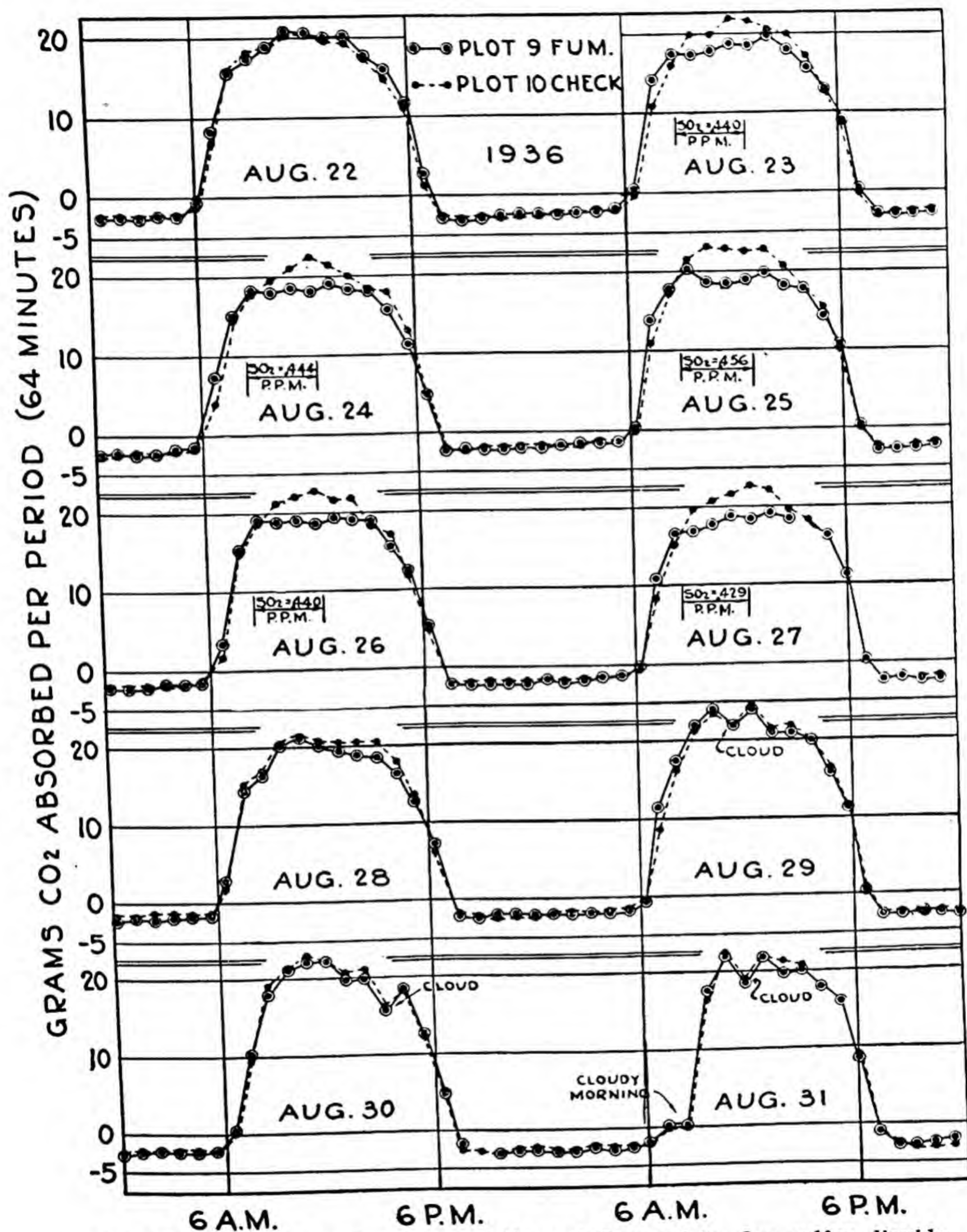


FIG. 2.13—The CO₂ exchange of alfalfa as influenced by five sulfur dioxide fumigations.

the curves diverged again slightly. A careful analysis of the data indicates that this prolonged fumigation at the low concentration produced no deleterious effect upon the growth of alfalfa, and for about half the time, a slight stimulation is suggested.

THE CO₂ BALANCE SHEET NET ASSIMILATION

In the 1935 and 1936 experiments, a number of plots of alfalfa were measured continuously for periods up to 56 days. Night respiration values were subtracted from the assimilation during the daytime to give net assimilation. As these crops were grown on soil, it was impossible to distinguish between root respiration and top respiration at night. For this reason, day respiration of the tops and roots could not be accurately calculated, and it was generally not worthwhile to try to estimate total photosynthesis.

It is probable that the CO₂ evolved in root respiration was accounted for in the exchange totals, as it diffused slowly from the soil. This would decrease the apparent assimilation in the daytime and increase the night respiration, but give correct net assimilation values. On the other hand, CO₂ derived from decaying organic matter or microbiological activity in the soil would reduce the net assimilation. This factor evidently was not large in these experiments, because the night respiration was not excessive, but rather was comparable to the night respiration observed in later sand culture experiments. Katz, Ledingham, and Harris (9) report night respiration values that seem to be unduly large compared with apparent assimilation, probably because of CO₂ derived from the rich, light soil on which the experiments were conducted.

Without making any allowance for CO₂ derived from roots or the soil, balance sheets have been constructed for the different crops of alfalfa in 1935-36 which indicate that 50 to 79 per cent of the net assimilation during the summer months is accounted for as top growth of the plants. However, a late season's crop which was measured from the time it was 60 per cent grown on August 24, 1936, until harvest on October 5, and another similar experiment in September, 1936, indicate that only 16 per cent of the net assimilation during this period was accounted for as top growth, the remaining 84 per cent presumably being translocated to the roots.

In 1940 and 1941, alfalfa was grown in the sand culture equipment. Four plots were measured over the major portion of the nine crops during the two years, except that on two of the plots, the 1940 first crop was measured on a few days only. There were short periods at the beginning of each crop when the plants were

TABLE 2.1
NET ASSIMILATION OF CARBON DIOXIDE, EXPRESSED AS EQUIVALENT DRY MATTER, BY NINE CROPS OF ALFALFA ON FOUR LOW SULFUR PLOTS AS COMPARED WITH THE CORRESPONDING YIELD DATA

Crop	High nutrient level ¹			Low nutrient level ²		
	Check plot		Fumigated plot	Check plot		Fumigated plot
	Net Assim. (A)	Yield (B)	B — A	Net Assim. (A)	Yield (B)	B — A
1940.....	(Kg.)	(Kg.)	(%)	(Kg.)	(Kg.)	(%)
First.....	(0.24)*	0.04†	..	(0.32)*	0.09†	..
Second.....	0.63	0.34	54	0.84	0.40	48
Third.....	1.09	0.66	61	1.43	1.12	78
Fourth.....	1.18	0.58	49	1.62	1.14	70
Total	3.15	1.62	51	4.21	2.75	65
Winter Resp...	-.27	-.27
1941						
First.....	2.56	2.59	101	2.60	2.82	108
Second.....	1.21	1.20	99	1.52	1.45	95
Third.....	1.70	1.39	82	2.28	1.82	80
Fourth.....	1.73	1.31	76	2.28	1.70	74
Fifth.....	0.93	0.74	79	1.14	0.81	71
Total	8.13	7.23	90	9.82	8.60	88
Total crops....	8.85	11.35	...
Roots and crowns....	2.86	2.72	...
Total crops and roots....	11.01	11.71	106	13.76	14.07	102

* Estimated.
† Low nutrient level on this crop.
‡ High nutrient level on this crop.

(1) 0.5 concentration Hoagland's solution.
(2) 0.2 to 0.02 concentration Hoagland's solution.

so small that it did not seem worth while to operate the analytical machines. For these periods an estimation was made by extrapolation of the assimilation curves, representing 6 to 9 per cent of the total net assimilation.

The data of Table 2.1 summarize the 1940-41 data on these four plots. The table includes values for the respiration of the roots during the winter when the plants were dormant, based on a few measurements of root respiration during this period. The net assimilation values are expressed as equivalent dry matter which contained 44 per cent carbon. Yield data for each crop are included

TABLE 2.2
NET ASSIMILATION OF CARBON DIOXIDE, EXPRESSED AS EQUIVALENT DRY MATTER, ON
FOUR PLOTS OF SUGAR BEETS, AS COMPARED WITH THE CORRESPONDING
YIELD DATA—1944

Nutrient Level	Y I E L D			Net Assimilation
	Roots	Tops	Total	
	(Kg.)	(Kg.)	(Kg.)	(Kg.)
Low.....	4.94	1.94	6.88	7.02
Low.....	4.75	1.77	6.52	6.72
High.....	4.15	2.78	6.93	6.70
High.....	4.41	2.39	6.80	6.75

in the table, and also the harvest weight of the roots and crowns. The total dry matter calculated from net assimilation agrees very closely with the yield data in all four plots, the maximum deviation being 6 per cent, thus indicating the accuracy of the exchange measurements.

During the first year, from 25 to 50 per cent of the assimilated CO_2 was transferred to the roots. In the second year, practically all the assimilated CO_2 from the first two crops appeared as top growth, but in subsequent crops an appreciable amount, 20 to 45 per cent, was transferred to the roots. The small, late season translocation to the roots in these experiments, as compared with 84 per cent in the 1936 experiments, is probably explained by the fact that in 1936 the crop was 60 per cent grown before assimilation measurements were begun.

Net assimilation and yield data for sugar beets in the sand culture apparatus, 1944, are summarized in Table 2.2. In this experiment the beets were planted late in May, but they did not begin

to make vigorous growth until the latter part of June. During the latter part of July the plants reached their maximum rate of assimilation, which then remained constant until the end of August. It then began to fall off gradually, until on the first of November the net assimilation was only half its maximum value. The table shows agreement within 3 per cent between net assimilation and yield. Total dry matter on the four plots was nearly the same, but those which had a low nutrient level produced relatively larger roots and smaller tops than the high nutrient plots. It is interesting to note that assimilation of CO_2 was not appreciably affected by a

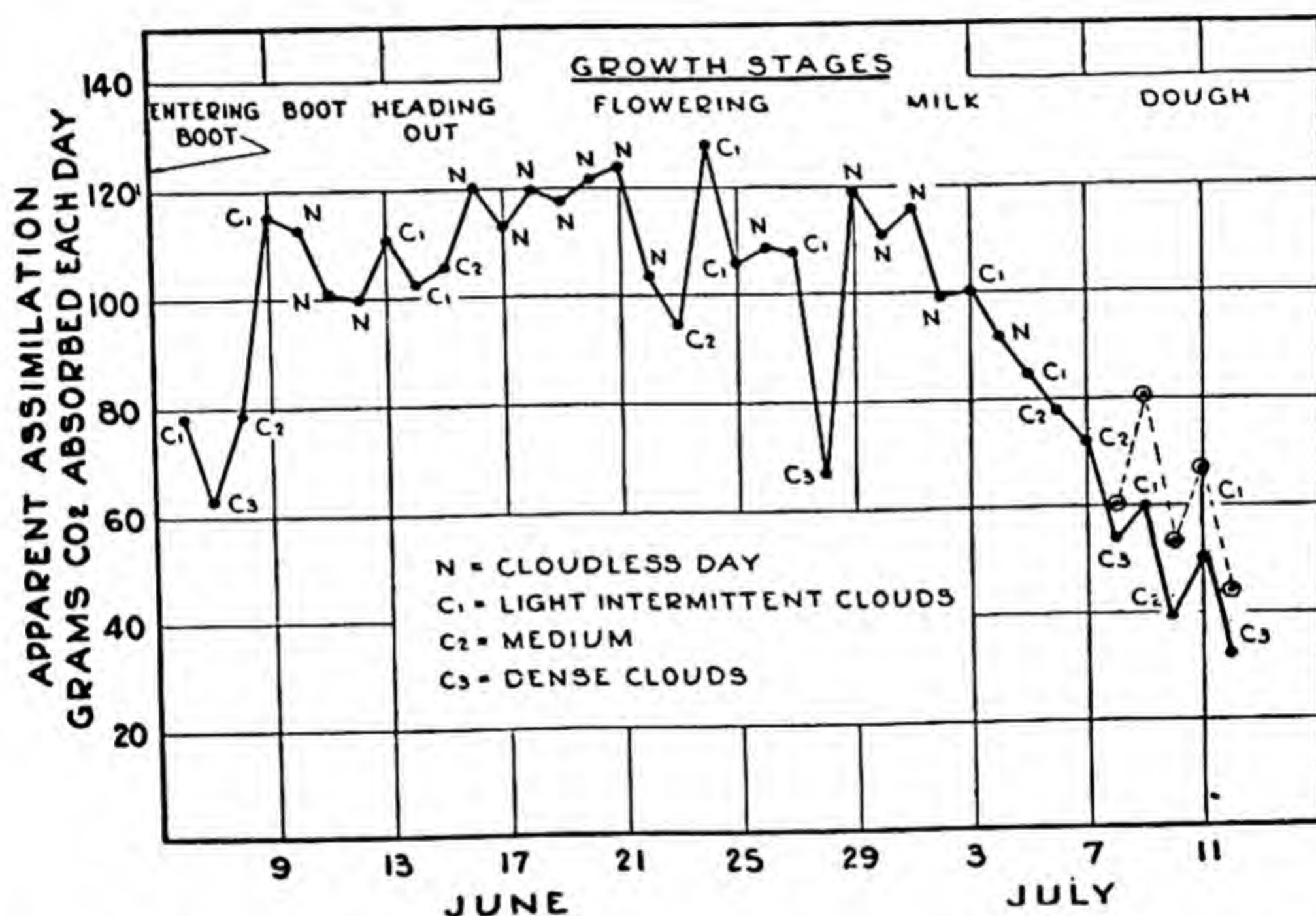


FIG. 2.14—Daily net assimilation of a plot of wheat in soil, at different stages of growth.

five- to tenfold difference in the concentration of the principal elements in the nutrient solution.

Net assimilation for wheat growing in soil is shown in Figure 2.14, covering the period from the early boot stage until ripening. There was a considerable increase in assimilation of the plants before the boot stage, associated with growth. Thereafter, the net assimilation remained approximately constant until the milk stage was reached, though a number of small fluctuations occurred during this period. After the milk stage, net assimilation decreased as the ripening process proceeded. Over the period covered by this experi-

ment, 83 per cent of the assimilated carbon dioxide was accounted for as top growth.

Figure 2.15 is from Heinicke and Childers (6) and gives the total monthly apparent photosynthesis, not including root respiration, of an 8-year-old apple tree, and also the average rates of assimilation during three periods of the day. Assimilation during the period from 7:30 P.M. to 9:30 A.M. is the excess of photosynthesis in the morning over respiration at night. The tree was in blossom from May 14 to 22. Its leaves developed rapidly between May 20 and June

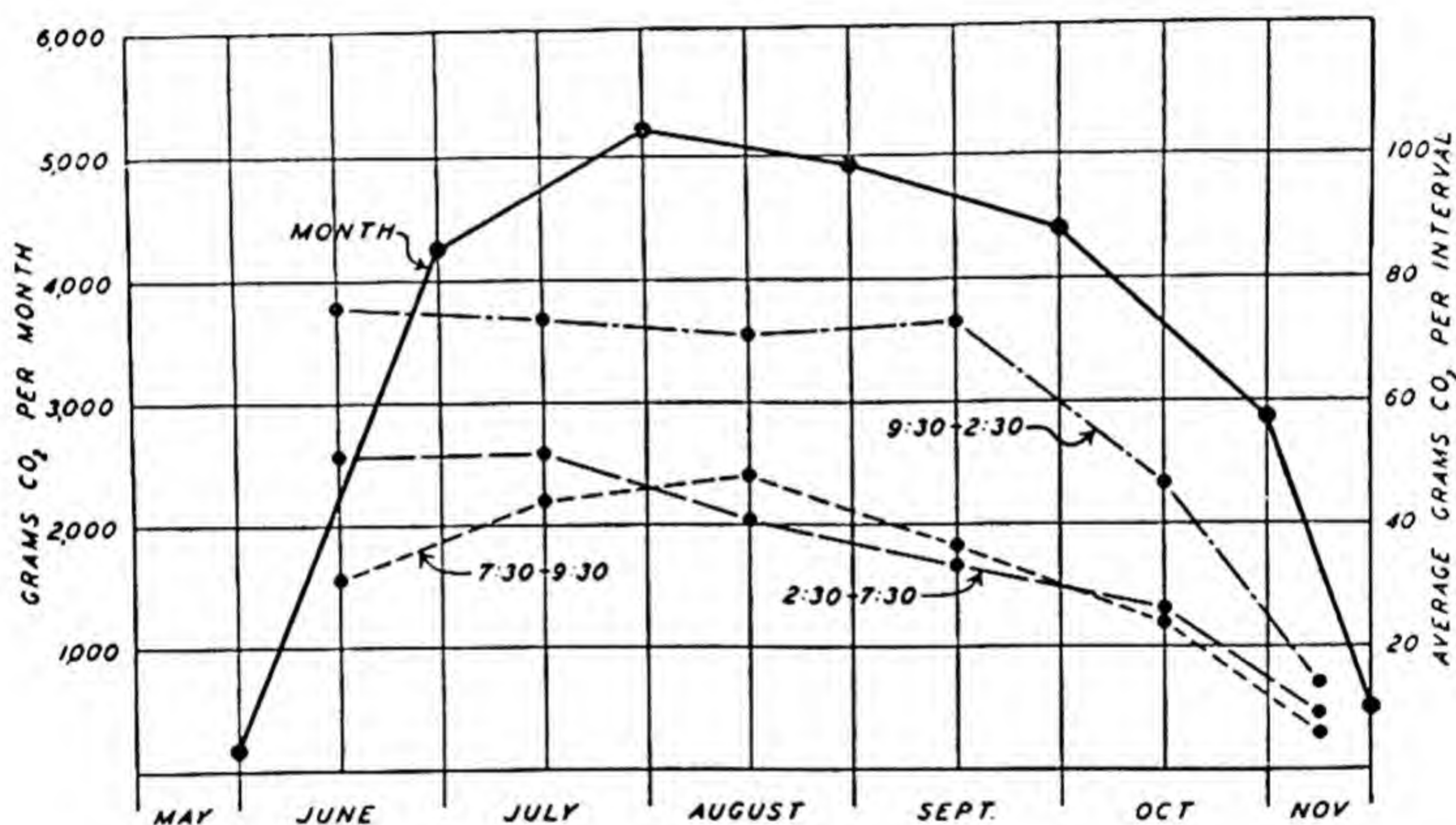


FIG. 2.15—Total apparent photosynthesis of an 8-year-old apple tree for each month of the growing season and average rate per 5-hour interval during day or 14 hour interval from 7:30 P.M. to 9:30 A.M. (after Heinicke and Childers).

10; thereafter new leaves were added slowly throughout the summer. Photosynthesis exceeded respiration for the first time on May 22 and apparent assimilation reached a maximum on June 25.

Leaf fall began in mid-October following freezing temperatures and was 90 per cent complete on November 17 when the experiment was terminated, at which time there was still measurable assimilation. Total CO₂ assimilation during the season was 22.2 kg., equivalent to 14.6 kg. of carbohydrate. The tree was cut down and the season's growth estimated, including the blossoms, fruits, leaves, shoots, and new growth on the old wood and roots. This amounted to 14.0 kg. The agreement seems satisfactory, considering the uncertainties of the estimation and the fact that root respiration

was not determined. The corresponding yearly net assimilation of sugar beets or second year alfalfa was 7 to 10 kg. of dry matter on plots of 36 square feet area, (Tables 2.1 and 2.2). The apple tree had a spread of 43 square feet and was 10 feet high.

TOP AND ROOT RESPIRATION

Table 2.3 gives a summary of top and root respiration, assimilation, and photosynthesis, calculated as a percentage of the photosynthesis of each crop, for alfalfa and sugar beets. A single plot of alfalfa and

TABLE 2.3

RESPIRATION AND ASSIMILATION OF ALFALFA AND SUGAR BEETS EXPRESSED AS PERCENTAGE OF THE TOTAL PHOTOSYNTHESIS OF EACH CROP

Crop	Respiration				Assimilation		Total Photo- synthesis
	Tops			Roots	Appar- ent	Net	
	Day	Night	Total	Total			
	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Alfalfa 1940							
First crop	(78) *	67	100
Second crop	(78) *	67	100
Third crop	15	8	23	16	79	61	100
Fourth crop	14	10	24	19	78	57	100
Alfalfa 1941							
First crop	11	5	16	19	81	65	100
Second crop	15	6	21	17	76	62	100
Third crop	15	6	21	15	78	64	100
Fourth crop	14	7	21	19	77	60	100
Fifth crop	12	8	20	29	73	51	100
Sugar Beets 1944							
Low nutrient.	12	6	18	11	82	71	100
High nutrient	14	6	20	13	78	67	100

*Arbitrarily assumed.

two plots of sugar beets are considered in the table. The other plots gave similar values.

The relative importance of the various factors is apparent. Top respiration and root respiration in alfalfa were approximately equal. Together, they account for 1/3 to 1/2 of the total photosynthesis. The root respiration percentage of alfalfa was highest in the fifth crop, when a large root system, poor top growth, and a protracted growing period combined to make it relatively more important than on the other crops. Root respiration in sugar beets was appreci-

ably less than top respiration, which in turn had about the same relative importance as in alfalfa. Net assimilation was correspondingly greater in beets than in alfalfa.

The root respiration values in the alfalfa experiments are shown in Figure 2.16. They indicate, in general, that root respiration falls to a low level when each crop is harvested, and rises con-

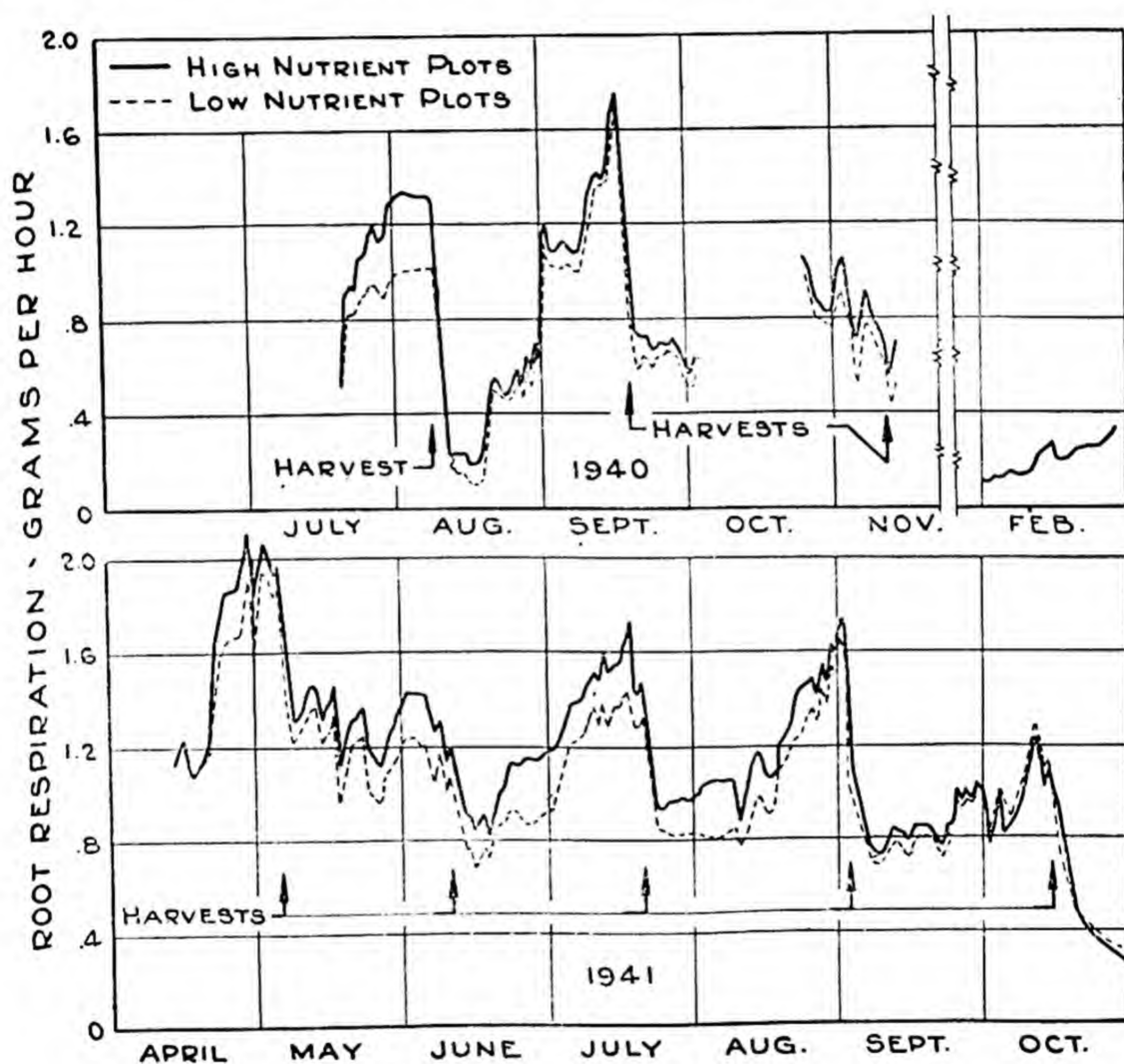


FIG. 2.16—Root respiration of alfalfa in 1940 and 1941.

tinuously as the crop grows. An exception to this relation occurred on the second crop in 1941, following the large first crop. Root respiration after harvest of the first crop fell to about 65 per cent of its value before harvest, then fluctuated somewhat without appreciable trend upward or downward during the time the second crop was growing. On some of the crops the root respiration values showed a sudden large increase, then remained nearly constant for about a week before making another sudden increase. These

periodic spurts seemed to be associated with clear days of high photosynthetic activity following a period of cloudy weather when the photosynthetic activity was low. This explanation is suggested by a curve representing a three-day running average of net assimilation, superimposed on the third crop 1940 root respiration curve (Figure 2.17).

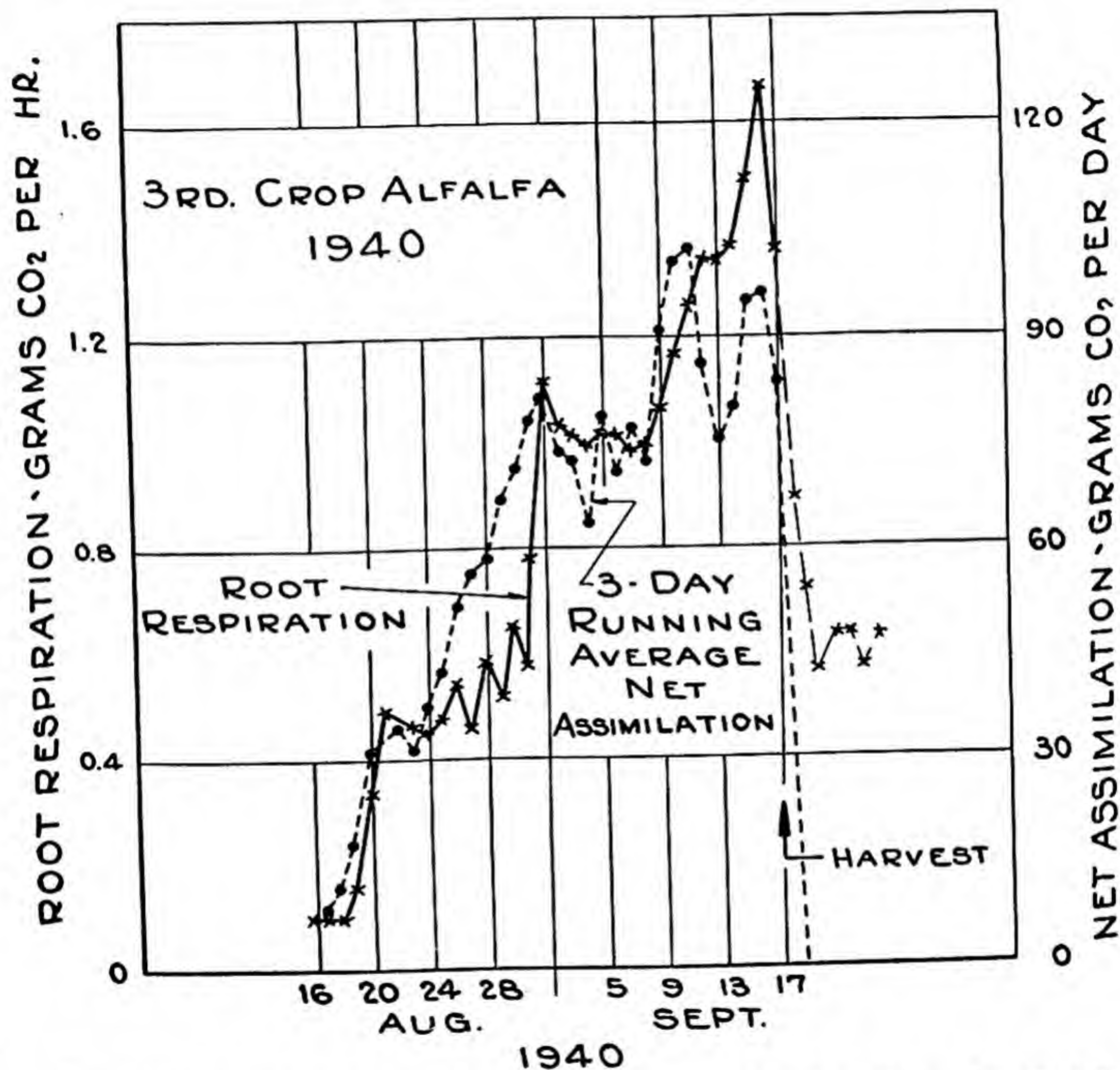


FIG. 2.17—Root respiration of alfalfa as compared with a three-day running average of the net assimilation.

The root respiration data suggest that root activity is closely associated with photosynthesis. It is necessarily true that root activity is dependent on the supply of the products of photosynthesis. Conversely, top activity requires root activity for the absorption and translocation of water and mineral solutes. Evidently, on the

1941 second crop, the roots had sufficient reserves at the beginning of the crop to maintain a high level of respiration.

TEMPERATURE COEFFICIENTS OF RESPIRATION AND PHOTOSYNTHESIS

It is well established that the temperature coefficient of respiration of many plants is represented by a Q_{10} of 2.0 or more. For example (18), night respiration of alfalfa growing in soil was measured during part of September when top growth was practically at a standstill, and consequently the quantity of vegetation was substantially constant. The respiration level increased about fourfold with a temperature rise from 0° to 20°C . Experiments conducted in sand cultures have given values of Q_{10} for the night respiration of alfalfa of 2.2 between 10° and 20°C .

Since photosynthesis is made up of light reactions, which have very small temperature coefficients, and dark reactions, which have temperature coefficients characteristic of ordinary chemical reactions, it is usually assumed that the over-all photosynthetic temperature coefficient is appreciable, possibly as large as Q_{10} of 2.0, except when the light intensity or the CO_2 is limiting. Noddack and Kopp (14) report Q_{10} values for *Chlorella* between 10° and 20° , of 2.26 for respiration and 2.07 for assimilation. Van der Paauw (21) has observed similar coefficients of respiration and photosynthesis with other green algae.

Emerson (2) found a sigmoid relation between temperature and the rate of photosynthesis of *Chlorella*, suggesting there were two or more independent, temperature controlled reactions. From 4° to 14° and 16° to 26° , the ratios of the photosynthetic rates in one experiment were 3.60 and 1.53, respectively. In other experiments the Q_{10} values of photosynthesis calculated from 5° to 6° temperature differences ranged from 25.0 at 1° to 6°C ., to 2.3 at 22° to 28° in *C. vulgaris*. The corresponding values in *C. pyrenoidosa* were 2.7 and 1.9. The marine alga *Gigartina* gave temperature coefficients similar to those of *C. vulgaris*. Evidently there are varietal effects to be considered.

McAlister (11) gives a graph for the respiration and photosynthesis of a young wheat plant at 12° and 31° , from which it may be deduced that the ratios of the rates of respiration, apparent assimilation, and photosynthesis are 3.1, 1.4, and 1.7, respectively, in this temperature range. The corresponding Q_{10} values would be 1.8, 1.2, and 1.3. The light intensity and the CO_2 concentration in this experiment are not stated.

In another experiment using air containing 0.3 per cent CO_2 , McAlister (12) obtained Q_{10} values of 1.0 and 1.9 for the photosynthesis of wheat at 16° and 26°C . when the light intensities were

500 and 2,000 foot candles, respectively. Decker (1) found that the apparent assimilation of red and loblolly pines was the same at 20° and 30°C., but fell off 45 per cent at 40°, whereas respiration doubled between 20° and 30° and increased 50 per cent between 30° and 40°. The temperature coefficients of photosynthesis of the two pines were 1.06 and 1.14 between 20° and 30°, and 0.72 and 0.66 between 30° and 40°C. The response of the two species to temperature was the same at 9,300 and 4,500 foot candles.

In most of the following experiments, no attempt was made to control either light intensity or the temperature. Accordingly, these two factors usually varied together and corresponded closely with the environment. When the light increased, the temperature usually increased also. Photosynthesis was estimated for each of the four 80-minute periods in the middle of the day between about 9:00 A.M. and 3:00 P.M., during the last week of each crop of 1941 alfalfa, and for a longer period on the fifth crop when the amount of top growth was practically constant. For each period, the average air temperature was determined from recording thermometers in the plant chambers, and the corresponding light flux from the pyrheliometer record. Leaf temperatures in the sun were doubtless higher, but were not measured.

The data for five crops of alfalfa in 1941 are given in Figure 2.18, in which the photosynthesis for each 80-minute period is plotted as ordinate against light intensity as abscissa. The data form somewhat rounded Blackman curves, in which the maximum photosynthesis was obtained when light exceeded about 0.75 cal./cm.²/min. on the first four crops and 0.55 cal./cm.²/min. on the fifth crop. These light intensities are equivalent to about 0.82 and 0.78 calories at normal incidence, and represent about 50 per cent of the normal maximum sunlight intensity in Salt Lake City, confirming the result of the 1935-36 experiments (18). Sugar beets and vegetative winter wheat gave curves similar to Figure 2.18. Light saturation in sugar beets occurred at about 0.70 cal./cm.²/min. on a horizontal surface, from August to October, and in wheat at about 0.85 cal./cm.²/min. in June and July.

The first four crops of alfalfa, though they varied in size, seem to fall reasonably close to the average curve and are not noticeably segregated. The fifth crop, which was considerably smaller than the others, also had less photosynthesis. Representative temperature data near some of the points on the curves show no relation between temperature and photosynthesis. It may, therefore, be concluded that with alfalfa in the field and also with sugar beets and wheat, from similar data, the temperature coefficient of photosynthesis is probably near unity.

These observations are in accord with the data of Heinicke and

Childers (6) on a young apple tree of bearing age. It may be noted that when their data covering the midday period from 9:30 A.M. to 2:30 P.M. are plotted against the light flux, curves similar to those in Figure 2.18 are obtained for each month. Light saturation occurred in June, July, and August, at about 0.7 cal./cm.²/min., and at about 0.65 cal./cm.²/min. in September and October. Assimilation in October and November was on an appreciably lower level

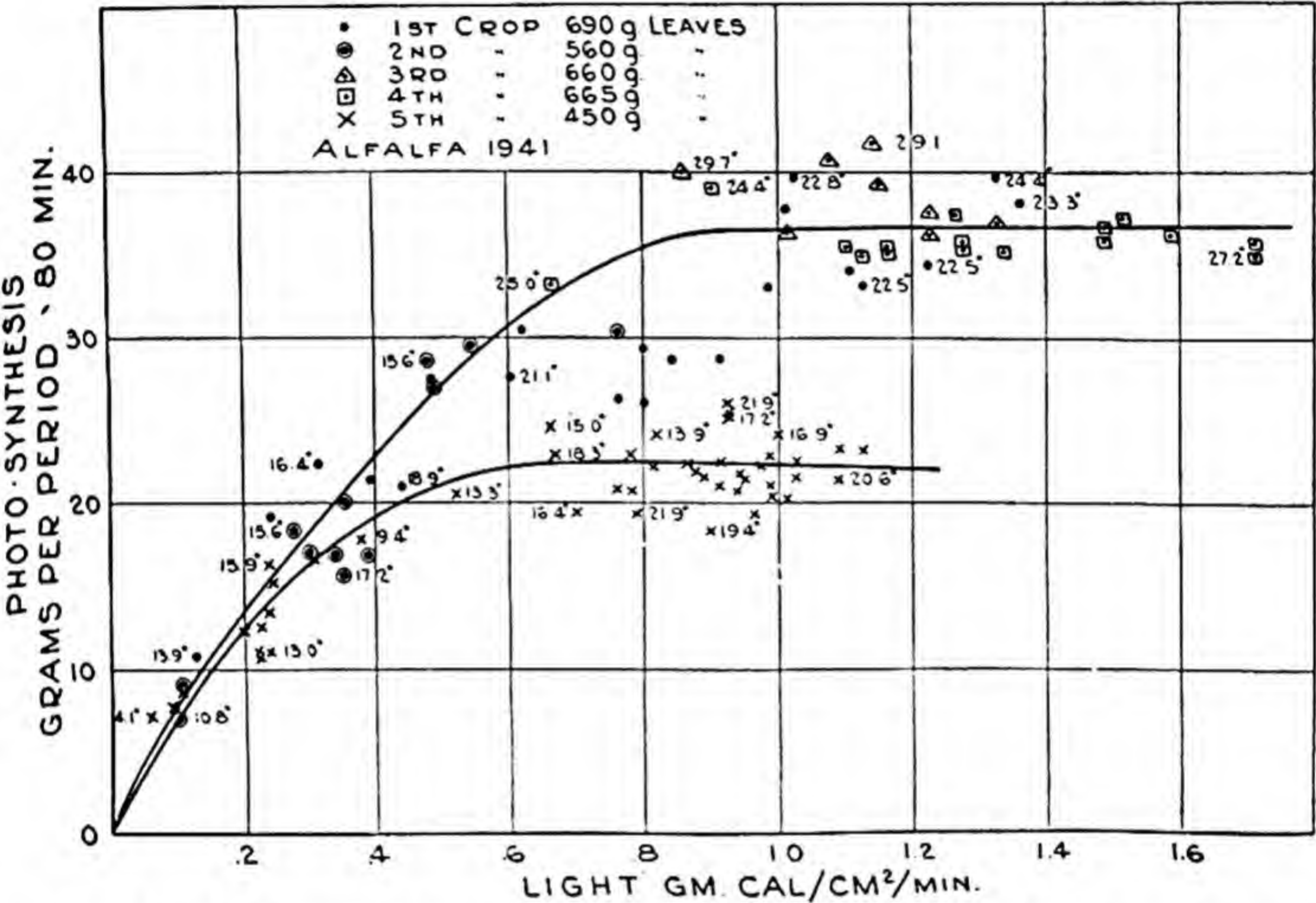


FIG. 2.18—Midday photosynthesis of alfalfa at different light intensities and temperatures, when the crops were fully grown.

than earlier, and light saturation probably did not occur in November. It is interesting to note that assimilation was consistently higher at a given light intensity in September than in other months; probably reflecting lower respiration due to lower temperatures. No other definite temperature effect on assimilation can be recognized in the data.

These observations are also in accord with those of Verduin and Loomis (22) who measured the photosynthesis of individual corn leaves and found light-photosynthesis relations like those in Figure 2.18. No definite effect of temperature on photosynthesis was apparent. Light saturation occurred at about 2,500 foot candles (0.4 cal./cm.²/min.), which is appreciably lower than the values for a plot or a tree, because the latter have many leaves that are only partially illuminated.

More definite data on these temperature relations were obtained in 1947 when one of a pair of similar plots was cooled by spraying water on the outside of the glass cabinets, which lowered the air temperature 2° to 12°C . Leaf temperatures were correspondingly reduced as indicated by thermocouples, but the effect of the spray on the light intensity was hardly measurable. Typical experiments with alfalfa at high light intensity are summarized in Table 2.4.

TABLE 2.4
THE TEMPERATURE COEFFICIENT OF PHOTOSYNTHESIS OF ALFALFA

Date (1947)	Average Air Temperature				Photosynthesis	
	Location	Plot D-4 $^{\circ}\text{C}$	Plot D-2 $^{\circ}\text{C}$	Difference $^{\circ}\text{C}$	Ratio D-4/D-2	Q_{10}
August	Outlet	28.0	21.5	6.5	$0.97 \pm 0.023^*$	0.95
August	Outlet	29.0	26.8	2.2	1.02 ± 0.045	1.10
September	Outlet	23.0	16.4	6.6	0.92 ± 0.035	0.89
September	Above plants	27.0	21.6	5.4	0.94 ± 0.033	0.90
September	Outlet	23.2	20.0	3.2	1.00 ± 0.061	1.00

* Standard deviation.

They indicate that the Q_{10} value for the photosynthesis of alfalfa was usually a little less than unity between 15° and 32° , suggesting a zero or slightly negative temperature effect under these conditions.

The same conclusion may be drawn from the observations on the equilibrium concentrations of CO_2 in an unrenewed atmosphere, where respiration and photosynthesis balance each other at high light intensity. Figure 2.19 shows that the CO_2 concentrations followed closely the air temperatures during the middle of the day when light intensity was nearly constant. The fact that Q_{10} of these CO_2 values is approximately 2.0 suggests that the temperature coefficient must be ascribed, in large measure, to respiration, and that the temperature coefficient of photosynthesis is therefore small. If, for example, the temperature coefficients of both processes were equal, the equilibrium concentration of CO_2 would be independent of the temperature.

Miller and Burr (13) have reported data on the equilibrium CO_2 concentration at high light intensity in an unrenewed atmosphere — some of which are independent of the temperature — suggesting that in those cases the temperature coefficients of respira-

tion and photosynthesis were approximately equal. It is not clear why some plants have a large temperature coefficient of photosynthesis, and others a small coefficient. Possibly the vigor of the individual plant is involved; also there may be varietal effects.

It is probably significant that in all the foregoing experiments which yielded Q_{10} values near unity, normal air or CO_2 -depleted air was used. McAlister's experiment (12) already referred to, in which

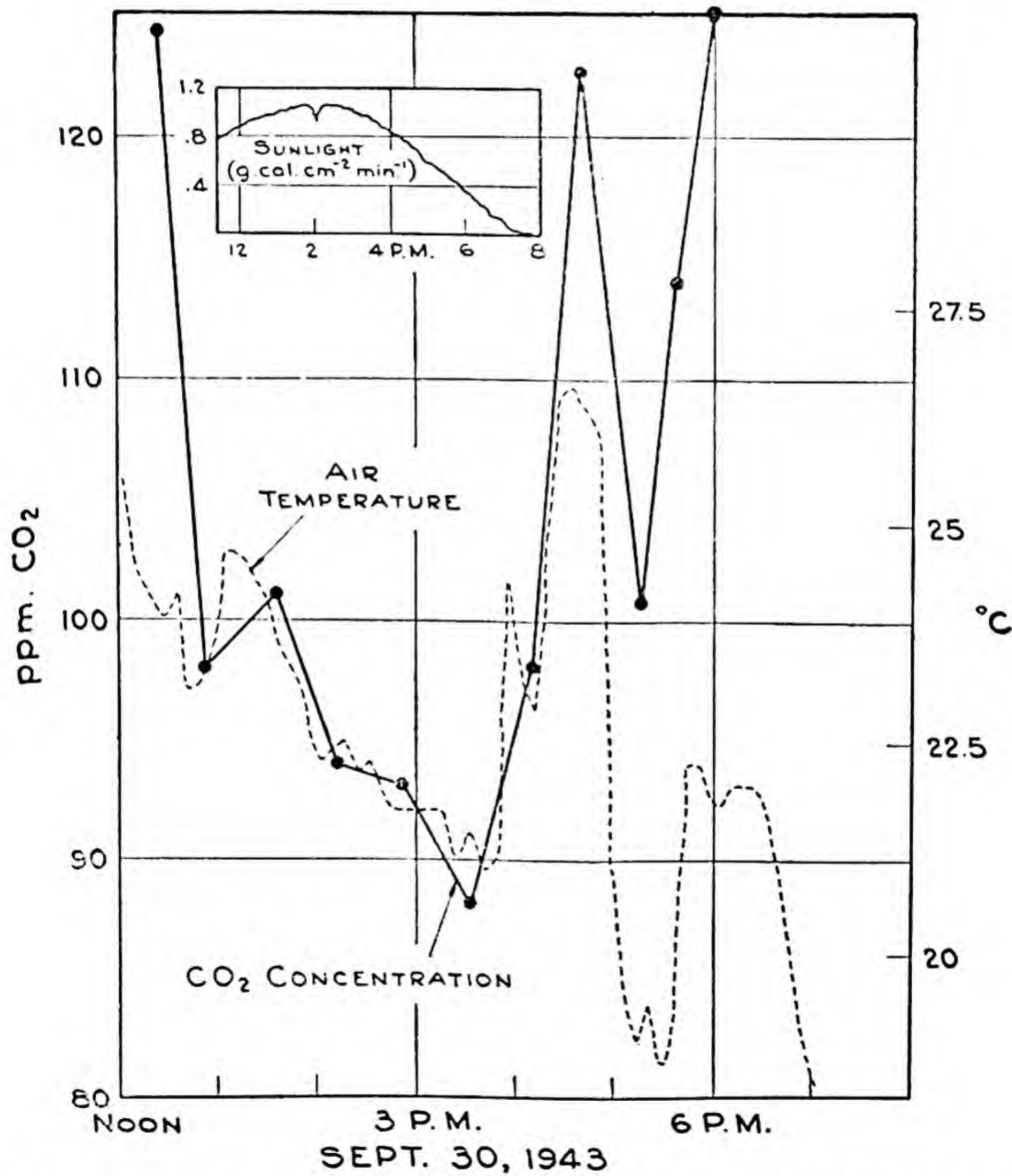


FIG. 2.19—Apparent CO_2 equilibrium in an unrenewed atmosphere between photosynthesis and respiration of alfalfa growing in the field, at different air temperatures and sunlight intensities.

CO₂-enriched air was employed, suggests that CO₂ concentration may be a limiting factor in modifying the temperature effect. This may also explain the large coefficient observed with the algae. The carbonate-bicarbonate buffer solution in which Emerson (2) made the photosynthesis measurements had an estimated CO₂ concentration of about 0.09 millimols per liter, whereas normal air has about 0.012 millimols per liter. Presumably at low CO₂ concentra-

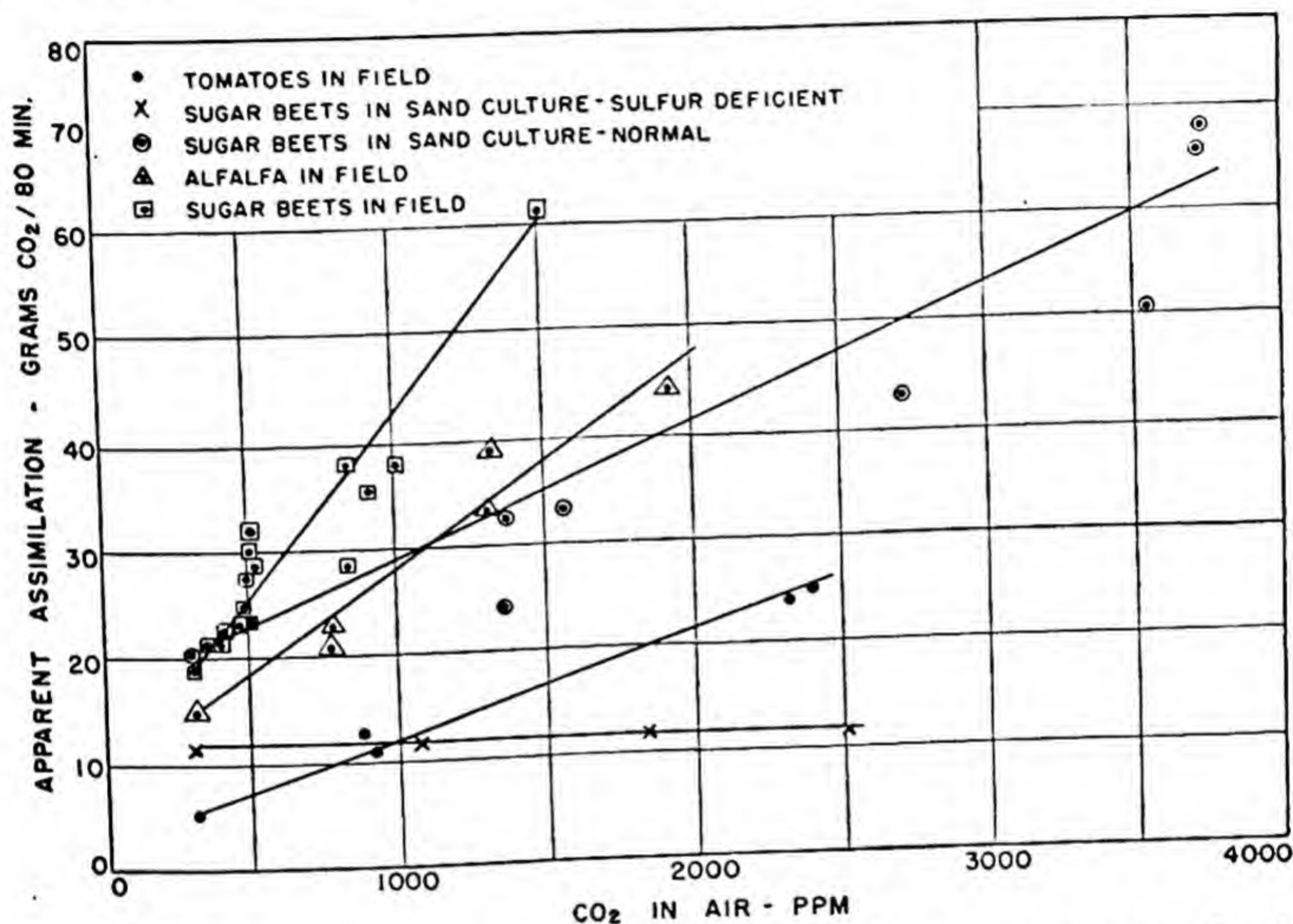


FIG. 2.20—Apparent assimilation by alfalfa, sugar beets and tomatoes, in air enriched with CO₂.

tion, the "light" reactions govern the rate of photosynthesis but at high concentration the "dark" reactions control.

CARBON DIOXIDE CONCENTRATION

A number of experiments have been carried out in which the concentration of CO₂ in the air has been increased. Figure 2.20 shows the relationship between apparent assimilation and CO₂ concentration for sugar beets, alfalfa, and tomatoes. With enrichment up to twelvefold normal air, there was nearly linear increase in apparent assimilation. The curves belong to a family in which the apparent assimilation is doubled by increasing the CO₂ concentration by the following approximate factors: tomatoes, 2.5–3.1; alfalfa, 2.7; sugar

beets in sand, 2.7; sugar beets in field, 2.5; sulfur-deficient sugar beets, ∞ .

Evidently there is a tendency for the different species to respond similarly to increased CO_2 concentration if the plants are normal, but the chlorotic, sulfur-deficient plants were unable to utilize the excess gas. The light intensity was high enough in these experiments to permit the assimilation of the highest concentration of CO_2 , and therefore was not limiting, as in the experiments of Hoover, Johnston, and Brackett (8). The latter obtained no additional assimilation in wheat, with a light intensity of 947 foot candles, when the CO_2 concentration exceeded about 1,200 ppm. At full sunlight intensity, extrapolation indicates that over 5,000 ppm. CO_2 could be assimilated. The slope of their linear CO_2 -assimilation curve (8, Fig. 7) yields a factor of 2.0, but the actual data depart from this curve, giving a factor of about 2.5, in agreement with the data in Figure 2.20.

It was noted that when the CO_2 enrichment treatments were discontinued, the photosynthesis of the plots returned quickly to normal, indicating little, if any, derangement of the normal photosynthetic mechanism. However, all these experiments were short-time treatments, lasting only a few hours at the most.

In 1946, CO_2 enrichment treatments were applied to tomatoes in which the concentration was increased approximately tenfold from 9:00 A.M. until 3 to 6 P.M. daily for about two weeks. As a result of these treatments, necrotic areas developed on many of the leaves, particularly on the plots which had a low general level of the elements in the nutrient solution. These lesions increased to an alarming degree, but when the treatments were discontinued, the plants developed new leaves and soon became normal in appearance again. A second two-week treatment, after a lapse of one month, caused a repetition of the effects, strongly suggesting that the CO_2 treatment caused the leaf injury. Dry ice was the source of CO_2 in this work. The actual cakes used were not analyzed, but the manufacturer claimed that the material was free of sulfur compounds and ethylene, and subsequent analyses supported the claim.

Immediately following the second treatment, a careful estimate was made of the leaf destruction. The plots with a low nutrient level, but adequate sulfates, had 25–30 per cent necrotic leaves in addition to 15–25 per cent chlorotic and partly necrotic leaves. On the plots with an adequate nutrient level, these values were 10–15 per cent and 8 per cent, respectively. There were only 1–3 per cent of necrotic leaves on the corresponding plots not treated with CO_2 , but there were 3–20 per cent of chlorotic leaves. While the necrotic effects were evidently due to the CO_2 enrichment of the air, it is probable that the chlorosis was largely due to other causes. Sub-

sequent CO_2 assimilation in normal air was reduced to a somewhat greater extent than would be expected from the leaf lesions. For example, the comparable midday apparent assimilation levels on one of the low nutrient plots, before and after the two enrichment periods, were as follows: 11.5, 6.0, 13.5, and 6.5 gm. CO_2 per 80-minute period. Presumably the photosynthetic apparatus of some of the uninjured leaves was overtaxed, particularly if adequate mineral nutrients were not available.

In another series of experiments such as those represented by Figures 2.11 and 2.19, a minimum CO_2 concentration in the air has been sought in an unrenewed atmosphere. With CO_2 from such sources as air leakage and root respiration excluded, and with full sunlight and a temperature of 15° , a plot of sugar beets established its equilibrium between respiration and photosynthesis at 40 ppm. This concentration agrees fairly well with the value estimated from the rates of respiration and photosynthesis in normal air.

DISCUSSION

The crop plants considered in this chapter exhibit a definite pattern of CO_2 exchange when growing under natural conditions, without undue stresses such as drouth or mineral deficiency. Even the chlorotic, sulfur-deficient plants had assimilation curves like the normal plants, but with less activity. The course of the assimilation curve was determined primarily by the light intensity. Temperature was certainly a minor factor and was effective principally through its influence on respiration, rather than on photosynthesis. This may not be the case in CO_2 -enriched air.

Transpiration likewise seems to be a secondary factor in photosynthesis. The diurnal transpiration curves (18) resemble closely the CO_2 exchange curves. Variations in transpiration are due primarily to the effect of temperature on the vapor pressure of water, and also to the rate of air flow over the vegetation. The apparent correlation between transpiration and photosynthesis is therefore fortuitous, since light and temperature usually vary together. Transpiration lowers the temperature of the leaves and this should raise apparent assimilation by reducing the respiration.

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3.

Products of Photosynthesis

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IN THE broadest sense all the organic materials in green plants may be considered to be the products of photosynthesis. These materials arise either directly from photosynthetic action or by transformation of the products resulting therefrom. If it were possible, we would like to distinguish between the products which come directly from photosynthetic action and those which are elaborated by subsequent metabolic processes and to limit our discussion chiefly to the former, but a clear-cut distinction between the two is well-nigh impossible. The two processes are so inter-related and so closely integrated that whatever division between them is made at the present time is more or less arbitrary.

The nature of the products of photosynthesis has been deduced largely from four types of observation: the quantitative aspects of the exchange of gases during photosynthesis; the composition of photosynthetic organs; the variation in composition of photosynthetic organs in periods of light and darkness; and the identification of labeled compounds arising from photosynthetic action in the presence of isotopic tracers. Worthwhile information has also been gained by special methods which have been little used; the heat of combustion method, the use of albino plants, and the method of reciprocal grafting.

Inasmuch as the photosynthetic process manifests itself largely through material change, it can only be completely understood through knowledge of the transformations of materials which occur within the photosynthetic organs. There is a great mass of information available on substances which occur in photosynthetic organisms, but little is known about the dependence of these substances on the photosynthetic process. In this chapter, whatever facts have been selected have been chosen so as to throw light on the material transformations associated with the process of photosynthesis.

GASEOUS EXCHANGE AND THE PRODUCTS OF PHOTOSYNTHESIS

One of the products of the photosynthetic process is oxygen. The liberation of this gas has been demonstrated by many methods both chemical and physical. One of the latest of these is by use of heavy oxygen, O^{18} . Heavy oxygen has been particularly useful in determining from what source the oxygen of photosynthesis is derived. In one experiment, *Chlorella* cells were suspended in a solution containing carbonate of ordinary isotopic composition dissolved in water rich in O^{18} . The oxygen evolved by illumination of the cells contained the same ratio of oxygen isotopes as the water which was employed. This indicated that the oxygen came exclusively from water (35, 64, 107). In another experiment performed by Tamiya and his associates (147), only two-thirds of the oxygen came from the water. Both experiments indicate that the oxygen produced by photosynthesis is largely, perhaps exclusively, derived from water; however, if Tamiya's results are correct, compounds besides water (according to Tamiya, CO_2) participate in oxygen evolution.

Inasmuch as two molecules of water are required to form one O_2 molecule, and since the ratio of O_2 liberated to CO_2 consumed is approximately unity, it is clear that the reaction is more complicated than that represented by the commonly accepted photosynthesis equation:



In order to account for the results obtained with isotopic oxygen the following equation should be used to satisfy the stoichiometry of the reaction:



The mechanism necessary to account for the stoichiometric relationships probably involves partial reactions in which the liberation of O_2 and the reduction of CO_2 take place in independent steps. One step would be the splitting of water in which oxygen is liberated and a reduction product is formed. The other step would be the reduction of CO_2 by the reducing agent formed. Such a mechanism has received some support from experiments on the illumination of isolated chloroplasts (cf. 54). When chloroplasts are suspended in water enriched in O^{18} and illuminated, the chloroplasts liberate oxygen of the same isotopic composition as the water which is used (57) and simultaneously reduce certain oxidants which are present in solution (5, 39, 54, 58, 140), but no carbon is fixed by reduction of CO_2 (22). However, evidence in support

of the production of an independent agent capable of reducing CO_2 has come from the following experiment. Algae, which are illuminated in the absence of CO_2 and then admitted to CO_2 in the dark, assimilate the gas into reduced carbon compounds much more rapidly than they do without previous illumination (28).

The intermediates responsible for oxygen evolution and for CO_2 reduction are, perhaps, the most sought-after intermediates of the photosynthetic process.

During photosynthesis, the volume of oxygen liberated usually approximates the volume of CO_2 absorbed. The ratio of these volumes, known as the photosynthetic quotient, ordinarily has a value near unity (144, pp. 332 ff.). This fact has been taken as evidence for the production of carbohydrate as the chief product, if not the sole product, of photosynthesis. From the photosynthetic quotients calculated for the formation of different organic products—formic acid, 0.50; carbohydrate, 1.00; protein, 1.25; and fat, 1.41—it is obvious that small variations in the photosynthetic quotient may indicate differences in the organic products formed by photosynthesis. Variations in the quotient have been observed. The photosynthetic quotients found by Maquenne and Demoussy (75) averaged about 1.03. This value could indicate the formation of as much as 12 per cent protein. Of special interest have been the photosynthetic quotients for diatoms because of their ability to synthesize fat. The values found for *Nitschia closterium* and *N. palea* are close to 1.05 (10). The diatom, *N. dissipata* has a quotient for O_2/CO_2 of 1.1 (138). These quotients correspond to the formation of material more reduced than carbohydrate and less reduced than fat. This photosynthate very likely possesses a level of reduction close to that of the whole mass of cellular material. The presence of highly reduced material in these organisms, *N. palea* and *N. dissipata*, is also evidenced by the low values of their respiratory quotients.

Photosynthetic quotients which differ from unity to an even greater extent have been noted in certain cacti. The values given for *Phyllocactus* are from 1.18 to 1.56, and for *Opuntia* from 1.12 to 2.28 (144). These "abnormal" values may be caused by photosynthesis of the CO_2 produced by respiration and retained by the fleshy photosynthetic organs of these plants, rather than by differences in the chemistry of the process. However, there is some evidence that the chemical transformations in these fleshy organs may differ from those in thin leaves.

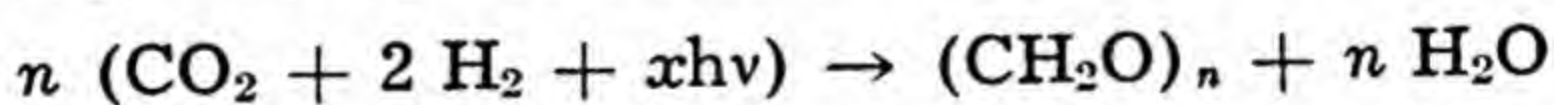
Nutrition affects the photosynthetic quotient. *Chlorella* cells cultured with nitrate gave higher values than when cultured with ammonium salts. The values observed for nitrate cultures were 2.0 at 40 fc. light intensity, and 1.25 at 600 fc. Cultured in

ammonium salt solutions the value was 1.11 at both light intensities (85). Since the photosynthetic quotient in the presence of nitrate is exceptionally high, some of the oxygen may have been derived from photochemical reduction of nitrate. (cf. 139). It may be, however, that the composition of the photosynthate differed considerably in the presence and in the absence of nitrate (cf. p. 78). These results show that light intensity affects the photosynthetic quotient, an effect supported by Barker's experiments on diatoms (10).

Even though the quotient be exactly unity, this would not prove that carbohydrates alone are synthesized, for as Spoehr (119-e) has pointed out, high values for proteins and fats and low values for acids might compensate each other so as to give an average value of unity.

There are certain circumstances in which the gaseous exchange of plants deviates greatly from the normal during different periods of the illumination. Emerson and Lewis (37) observed that *Chlorella* cells which had been kept for some time in the dark, produced a gush of CO_2 when illuminated (cf. 67). It should be pointed out that the gush of CO_2 might arise from a decarboxylation which of itself could be an energy storing reaction. Fluctuations in the uptake of CO_2 during photosynthesis have been observed by Kostytschew *et al.* (68) during the course of the day. In some instances CO_2 was evolved. What influence the abnormal gas exchange has on the organic products formed is still to be determined.

The gaseous exchange of *Scenedesmus* is at times most unusual. Under some circumstances, *Scenedesmus* when illuminated in an atmosphere containing both hydrogen and CO_2 , absorbs both gases and in the ratio of two molecules of hydrogen to one of CO_2 . The equation representing the stoichiometric relations is:



where $h\nu$ designates light energy and CH_2O , carbohydrate. The gaseous exchange indicates that carbohydrate is the organic product formed. When the reduction of CO_2 is suppressed, hydrogen is evolved on illumination of the organism. This instance of the evolution of hydrogen on illumination of a photosynthetic organism is exceptional (44, 45).

In summary, it may be said that the usual close approach to unity of the value for the photosynthetic quotient is in agreement with the concept that carbohydrate is the principal organic product of photosynthesis. The variations from unity suggest that other organic products are formed under certain circumstances. Correlation of the gaseous exchange with the organic products formed in the unusual cases is still incomplete.

NATURE OF THE ORGANIC MATTER FORMED IN PHOTOSYNTHESIS
OCCURRENCE AND DIURNAL FLUCTUATION OF ORGANIC SUBSTANCES
IN LEAVES AND ALGAE

In order to understand the material changes brought about by photosynthesis, it is necessary to know what substances exist in photosynthetic organisms and how they vary during periods of light and darkness. Inasmuch as the materials differ so greatly in different plants, the photosynthetic process itself may vary in detail in the different organisms. In order to assess this variation it is essential to know the distribution of various substances among autotrophic organisms, especially among those parts of plants concerned with photosynthesis.

In the material that follows, only the most superficial survey of this field is made. For more complete information, reference should be made to the larger works, for example: Klein, (65); Onslow, (90); Wehmer, (141); and Wiesner, (143).

Carbohydrates and Carbohydrate-like Substances

There is a wide variation in the nature of the carbohydrates and carbohydrate-like materials found in various photosynthetic organs and organisms. This is not surprising in view of the different morphological structures represented—single-cell organisms, multicellular organisms; thick leaves, thin leaves; aquatic leaves, xerophytic leaves, and so on.

Almost without exception, when pentoses and methyl pentoses occur in photosynthetic organs and organisms in the free state, they occur in very small quantities (47-a, 100, 119-b). However, in *Sedum praealtum* the pentose accounts for 10 per cent of the aldose content (16-b).

The hexoses, glucose and fructose, are widely distributed, but mannose has failed of detection in the leaves of forty-two species of plants (31-b).

Although glucose appears to be widely distributed in the higher plants, it is present only in small quantities in some algae, if present at all (100). For example, immediately following photosynthesis, free reducing sugar is present to the extent of less than 0.5 per cent in *Scenedesmus obliquus* (21) and after a vigorous period of photosynthesis in which *Nitzschia palea* should have accumulated an amount of sugar equivalent to 9.5 mg. of glucose (calculated from the oxygen evolved), no distinguishable carbohydrate could be detected by analytical means (10).

The behavior of reducing sugar during periods of light and darkness is variable. In the narcissus leaf, Barton-Wright and Pratt (14) observed a general decline in the amount of hexose sugars

during the day as is shown in Figure 3.1. In the leaves of the snowdrop, Parkin (92) also noted a "slight diminution in hexose, with a corresponding increase in sucrose, on the first exposure of a darkened leaf to light." However, the hexose sugars under some circumstances increase immediately on illumination (cf. Fig. 3.2

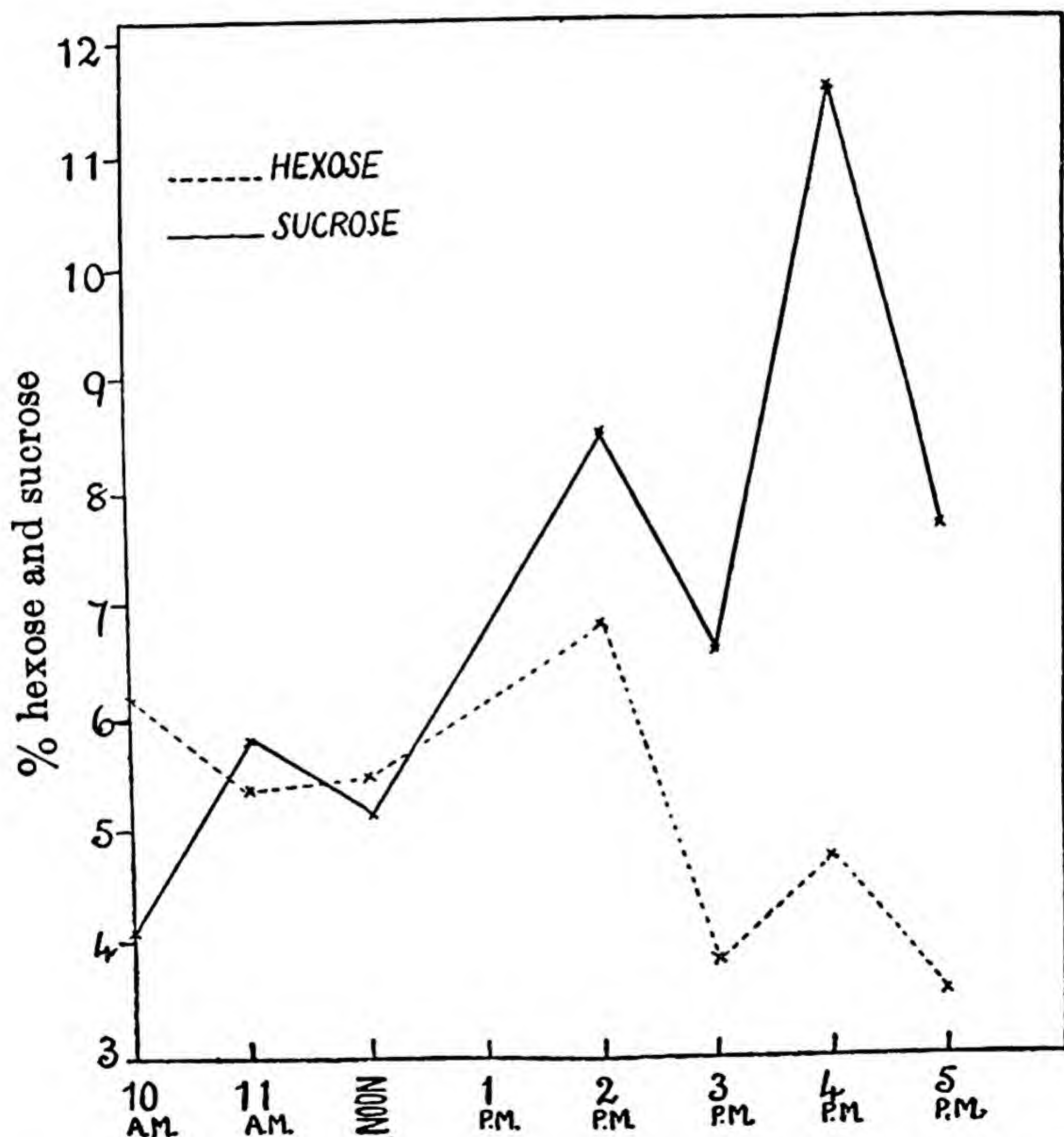


FIG. 3.1—Hourly variation of the hexose and sucrose content of narcissus leaves (Barton-Wright and Pratt [14]).

[31-a]). From these and other experiments it is evident that the behavior of the hexoses is not consistent. This makes it difficult to understand their place in the scheme of carbohydrate synthesis during photosynthesis.

In most experiments on the occurrence and diurnal fluctuations of hexoses, no distinction has been made between glucose and fructose. However, in the experiments of Parkin on leaves of snow-drop (*Galanthus nivalis*) fructose predominated over glucose in forty-seven out of fifty-two analytical determinations. Parkin con-

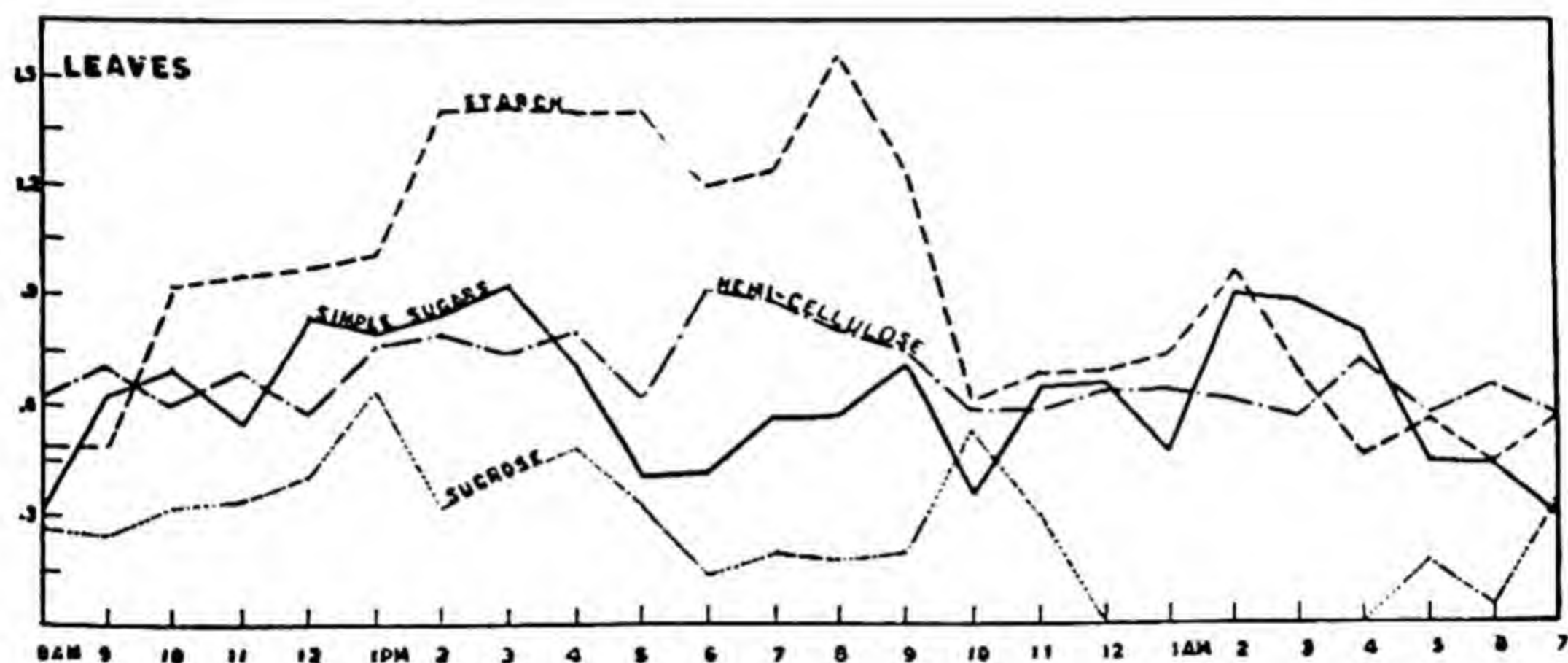


FIG. 3.2—Hourly variation of the carbohydrates of sunflower leaves under natural conditions (Clements [31a]). Ordinate: percentage of fresh weight.

cluded that these sugars arose from the hydrolysis of sucrose. A similar conclusion was reached by Brown and Morris (23) from their experiments on *Tropaeolum majus* (cf. 117-b).

An unusual ketose, sedoheptose, has been detected in the acid plants *Sedum praealtum* (16-b) and *Bryophyllum calycinum* (97-a). This sugar accounted for the entire ketose content of *S. praealtum* leaves examined by Bennet-Clark. Remarkable diurnal fluctuations in the quantity of this sugar have been observed by both of these investigators (97-b).

As Spoehr (119-e) has pointed out, "It is in the form of the oligosaccharides, glucosides, and polysaccharides that the plant kingdom presents a remarkable variety. These are composed not only of *d*-glucose and *d*-fructose, but include pentoses, methyl pentoses, *d*-galactose, *l*-galactose, *d*-mannose, *d*-sorbose, and heptoses. Many combinations of these and also these units with uronic acids, go to make up the tremendously complex compounds which we find primarily in the structural and reserve food tissues."

Sucrose is probably the most widely distributed of all the disaccharides. "There is much evidence to show that this sugar is very widely if not universally distributed in the Flowering Plants. It occurs also in the Vascular Cryptogams, but has been less studied in these. Its presence in the Mosses has been asserted" (92).

Brown (21) has reported that sucrose is the major constituent in the sugar fraction of *Scenedesmus obliquus*. From the large diurnal change exhibited by sucrose (cf. Figs. 3.1 and 3.2) under nearly all circumstances, it is clear that this compound plays an important role in and is one of the first carbohydrates to be formed by the photosynthetic process. Maltose has been reported in the leaves of the nasturtium (23) and of mangold (29). The results of these investigators indicate that this disaccharide fluctuates diurnally. However, the more recent analyses of Van der Plank (131) cast some doubt on the occurrence of this sugar. Trehalose exists in free form in several of the Florideae, and constitutes 10 per cent of the dry weight of *Rhodymenia palmata* (71). The trisaccharide, raffinose, has been reported in the leaves of *Taxus baccata* (65).

Starch is the most common polysaccharide known to be intimately connected with the photosynthetic process. The starch in leaves is present chiefly in the chloroplasts and this suggests its close connection with photosynthesis. However, it is not formed in all photosynthetic tissue under ordinary conditions of photosynthesis, neither is it formed exclusively in photosynthetic tissue. Plants vary greatly in their ability to accumulate starch. Some leaves, e.g., tobacco, may accumulate upwards of 40 per cent of their dry weight as starch (121-a) whereas other plants, such as onion and snowdrop, fail to form starch even under conditions considered to be favorable for its formation (90). Starch has been isolated from leaves of several species of plants and the properties of the various samples recorded. Hydrolysis of the different samples yielded glucose almost quantitatively (121-a, -b).

Starch formation is not confined to the higher plants, for in many algae it constitutes the reserve food material (115). In an examination of the products of photosynthesis by *Scenedesmus obliquus*, Brown (21) found that the chief carbohydrate present was a starch-like polysaccharide, which is in agreement with earlier reports that the storage product of other Chlorophyceae is starch.

Starch exists in more than one form, notably as amylose (β -amylose) and amylopectin (α -amylose). Amylose is a polyglucose of molecular weight from 10,000 to 60,000, consisting of long unbranched chains of glucose molecules linked in α -configuration. Amylopectin has a molecular weight of from 50,000 to 1,000,000 and is formed from glucose molecules linked together in chains which are branched (52-b). The relative amounts of these two in leaf starches is still unknown. Such knowledge might prove valuable in determining the mechanism of starch formation in

leaves. When starch is formed in a leaf it frequently shows the greatest diurnal variation of any of the carbohydrates, e.g., Figure 3.2 (31-a, 82). The starch-like polysaccharide in *Scenedesmus obliquus* is the chief carbohydrate present after an extended period of photosynthesis (21).

The polysaccharide food reserves differ in different organisms, particularly in the algae. In some, starch is formed, but in others the carbohydrate food reserves differ from starch and are designated by various names: laminarin, paramylum, floridean starch, leucosin, and others not so well known. Except for laminarin, the exact nature of these compounds is not known.

Laminarin, found in various species of *Laminaria*, and considered by some authorities to take the place of starch in the fucoids, has been shown to be a polyglucose. The glucose units are combined by 1,3-glycosidic linkages which are of the β -type (13; cf. 52-c).

In many species of algae the polyglucose, laminarin, constitutes a very considerable portion of the dry weight. *Laminaria saccharina*, at times, contains 49 per cent (100). Definite proof of the immediate response of laminarin to illumination is still not available. However, it fluctuates diurnally to some extent and it disappears on storage in darkness (87).

Paramylum is the chief product of photosynthesis in euglenas. It does not give the usual tests for starch even though it has the same empirical formula and is hydrolyzed to glucose (128). Paramylum increases on illumination and decreases on darkening the algae by which it is formed. This is evidence for its photosynthetic origin, but the paramylum grains form in the cytoplasm outside the chloroplast, which suggests a strictly metabolic origination (84).

Floridean starch is a food reserve of the red algae. This carbohydrate isolated from *Lithothamnion calcareum* closely resembles glycogen. It gives a brownish-red color with iodine, is digested by pancreatic enzyme, and forms glucose on hydrolysis (33, 74-b). The dependence of floridean starch on photosynthesis appears to be indirect because this carbohydrate accumulates in grains separated from the chloroplast (74-a).

Leucosin is a food reserve of the Chrysophyceae which is accumulated in the cytoplasm (115, pp. 183, 188). However, it is reported to be the chief photosynthetic reserve material of certain algal forms. Nothing is known of its chemical structure, but it is produced by feeding glucose, sucrose, and maltose; therefore, it is assumed to be a carbohydrate.

Much physiological work needs to be done on these algal forms

to relate the formation of these "photosynthetic reserves" to photosynthesis. The determination of the chemical structures of the different reserve carbohydrates may open the way to a better understanding of the similarities and dissimilarities of the chemical processes producing them.

Polyfructoses are also present in leaves (3). The fructosan content of barley leaves increases as the total carbohydrate concentration increases. This relationship indicates the secondary origin of the fructosan and its function as a temporary storage product. The fructosan from barley leaves contains about 94 per cent fructose and 6 per cent aldose. The molecule consists principally of a chain of ten fructo-furanose units linked through carbon atoms 2 and 6. The most commonly known polyfructose, inulin, has been found in leaves although it does not seem to be a common constituent of leaves (65, II, p. 1884; 130, p. 326).

Even though free mannose is absent from leaves, complex carbohydrates composed of mannose (mannans) may be present (141, p. 81). A water-soluble polysaccharide has been isolated from perennial rye grass which is composed of galactose and glucose and yields more than 30 per cent ash. The ash is calcium sulfate, which indicates the presence of a sulfuric acid ester of a polygalactose containing glucose residues. While polygalactoses containing sulfuric acid residues are common in algae, this is the first one isolated from the higher plants (78).

There are many substances in the cell walls of leaves which are of carbohydrate nature. Primary among these is cellulose. The blades from several species of grass contain about 20 per cent of their dry weight as cellulose (27-a). Cellulose is composed of glucose units linked together through 1,4-positions with β -glucoside configuration. In general structure, it resembles starch except that starch has the α -glucoside configuration (52-b). Although closely related chemically to starch, it does not show appreciable diurnal fluctuation (60).

Cellulose is present also in certain algae. In the brown algae and the Florideae the amounts present are considerably less than in the higher plants. In many algae the cellulose portion of the cell wall is a minor constituent. In the Bangiales, cellulose apparently is absent (83). From what is known about its structure the cellulose in algae is the same as the cellulose of the higher plants.

The other cell-wall constituents in the leaves of higher plants are largely hemicelluloses and pectic substances. On the basis of dry weight, the hemicelluloses comprise about 2.65 per cent of the material of the bean leaf and 25.6 per cent of the maize leaf. The pectin, as calcium pectate, accounts for 7.74 per cent of the bean

leaf and 0.9 per cent of the maize leaf. Uronic acid rarely exceeds 25 per cent of the hemicellulose molecule, whereas it accounts for about 70 per cent of the pectin structure (27-b).

Buston has divided the hemicelluloses into three fractions: "A," a water-insoluble fraction, and "B" and "C," water-soluble fractions. These fractions differ in composition in different leaves. The A fraction of bean and vine leaves consists of hexosans; of grass, xylan; and of maize, much pentosan. The B and C fractions of bean and vine leaves consist of pentosans and uronic anhydrides; and of grasses, of pentosans, hexosans, and uronic anhydrides. A detailed analysis of cocksfoot leaves shows, for example: A, xylose and arabinose; B₁, xylose, arabinose, and galacturonic acid; B₂, galactose and arabinose; C₁, xylose, arabinose, and uronic acid (?); and C₂, galactose, arabinose, xylose, and galacturonic acid.

Bennett (15) has isolated polyuronide hemicelluloses from two grasses and found them to contain uronic acid, l-arabinose, and d-xylose. In these grasses they occur in the following proportions: sheep's fescue (*Festuca ovina*), 1:0.2:15.7; and sweet vernal grass (*Anthoxanthum odoratum*), 1:2.9:9.3.

Hemicellulose fluctuates during photosynthesis to some extent, as shown in Figure 3.2, and has been assumed to be in mobile exchange with the other carbohydrate components of the leaf and to be important as a food reserve material (31-a). Buston (27-b) found that when leaves were stored in the dark the hemicellulose A fraction remained approximately constant whereas the B and C fractions decreased. The decreases in these fractions were as follows: bean leaves, 38 per cent in 7 days; grape leaves, 66 per cent in 21 days; and maize leaves, 19 per cent in 19 days. The composition of the hemicelluloses differed after the periods of starvation; there was a loss of hexosan and pentosan and a gain in uronic anhydride. The stability of the A fraction seemed anomalous inasmuch as it was composed of hexosan. However, this anomaly may be explained by the fact that it is a galactan.

Little can be said concerning the relationship of pectin to photosynthetic action in leaves. According to the results of Buston there is little change in this substance brought about by starvation of the leaf.

The cell-wall constituents of algae are not known to vary significantly between periods of light and darkness. However, in many of the species of red and brown algae, these materials are so situated with respect to the active photosynthesizing cell, and form so large a part of the organic material of the plant, that they appear to be the chief products formed by the photosynthetic process.

It is the intercellular substance that composes the great bulk of the algal cell-wall material. For example, Bird and Haas (18) have found 60 per cent of the dry weight of certain *Laminaria* species to consist of alginic acid. This acid comprises from 20 to 25 per cent of the dry weight of most species of brown algae examined. So far as is known, alginic acid is a polymannuronic acid (83, 119-f).

The intercellular substance of the red alga, *Porphyra tenera*, comprises 35 per cent of the entire algal substance and is composed chiefly of a galactose-sulfuric acid ester (67 per cent galactose, 16.3 per cent organic SO_4). The galactose is made up of 0.9 *dl*-galactose + 0.1 *d*-galactose (83).

The best-known of these substances is agar-agar which has been obtained from many species of algae. It accounts for about 25 to 35 per cent of the air-dry weight of the red alga, *Gracilaria confervoides* (59).

Agar possesses a complicated and extraordinary structure. The chief constituent is a galactose-sulfuric acid ester (94). The molecule consists of nine *d*-galactose units linked through 1,3-positions and one *l*-galactose unit at the end of the chain linked through the 4-position. The *l*-galactose unit is esterified in position-6 with sulfuric acid. The occurrence of the *d*- and *l*-forms of a given sugar in the same molecule is unusual. This may give some clue to the metabolism of the sugars in the formation of the polysaccharides. In this connection it may be of significance that galactose is the only hexose that can be transformed into its optical antipode by interchange of the two functional end-groups—aldehyde and primary alcohol groups.

A galactan similar in structure to agar is contained in carrageen moss. The sulfuric acid group is attached in the 4-positions (52-c). Another galactose-sulfuric acid polysaccharide has been isolated from *Iridea laminarioides* which contains one sulfuric acid group for each galactose unit (51).

Many other cell-wall constituents exist in algae which vary in composition from species to species. A mucilage has been obtained from *Laminaria japonica* which contains 26.7 per cent fucose, 39.2 per cent glucose, 39.9 per cent sulfate (SO_4), and 4.4 per cent uronic acid. A mucilage from *Fucus evanescens* is a polymer of fucosemonosulfate (83). Fucoidin occurs in species of *Laminaria*, also in *Fucus serratus*, and *Ascophyllum nodosum*. It consists of a sulfuric acid ester of a fucose-uronic acid complex. The nature of the uronic acid is not known (18). From these examples it is clear that the cell-wall constituents of various photosynthetic organisms vary.

Sugar Alcohols

The reduction product of mannose or fructose, mannitol, is present in certain leaves. In *Veronica tourneforti* it comprises as much as 12 per cent of the dry weight (8). The chief occurrence of mannitol in photosynthetic organisms, however, is in algae, particularly in the Phaeophyceae. Mannitol is almost universally present in the brown algae, whereas it is absent from the red algae which have been examined (47-c). The percentage of the dry weight in certain species is as follows: *Aschophyllum nodosum*, 5.2; *Fucus serratus*, 6.5; *Laminaria digitata*, 6.8 (71). It attains the high values of 25.7 per cent in *L. saccharina*, 28.9 per cent in *L. flexicaulis* (100), and 36.7 per cent in *L. cloustoni* (19).

Mannitol behaves in many respects as though it were closely connected with photosynthesis. In celery (*Apium graveolens*) its amount depends upon the assimilatory surface of the plant and parallels the sucrose content. Furthermore, it increases in leaves in the light and decreases in the dark (89-a). But in leaves of *Gardenia* sp. which contain mannitol, the mannitol bears no visible relationship to the process of photosynthesis (8). Mannitol can be considered to be a reserve material for the fucoids (71). Fluctuation of the mannitol between day and night is small but on continuous storage of the algae in the dark the mannitol diminishes (87). Because of its ubiquitous occurrence in the fucoids it has been suggested that "mannitol, not sugar, is a primary photosynthetic product, which implies that the carbon metabolism of the fucoids is based on alcohol rather than on sugar" (47-c).

A compound of mannitol is present in *Pelvetia canaliculata* and its form *libera* which contains a number of mannitol residues, and is designated mannitan (47-a).

Other sugar alcohols occur in photosynthetic organs. Sorbitol has been found in leaves of several plants. It may represent 1.2 per cent of the "undried material" (127). The red alga *Bostrychia scorpioides* contains about 13.6 per cent, but no other species of Rhodophyceae examined contained this alcohol (47-b). Dulcitol has been obtained from leaves of *Evonymus europaeus* (89-b), from the red algae *Bostrychia scorpioides*, which contains about 8.3 per cent (47-b), and from *Iridea laminarioides* (51). A seven-carbon alcohol, perseitol, has been reported from the leaves of *Persea gratissima* (130). The cyclitols are found in the leaves of various plants and may have an important function in plant metabolism. Also a careful examination of their structural relationships to other carbohydrate constituents in leaves might give indication of the courses of metabolism in different species (4, 40, 130).

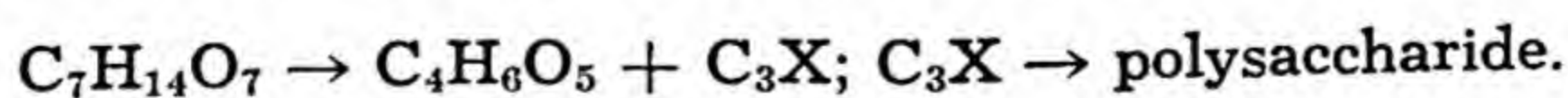
Organic Acids

In recent years the metabolism of carbohydrates and proteins, and photosynthesis itself have been shown to be closely bound up with the transformations of certain organic acids. Many of these acids or their salts have been identified as constituents of leaves and other photosynthetic organisms as a short summary of their occurrence shows.

Formic and oxalic acids are found in many leaves (65). Pyruvic acid accumulates in barley leaves when the carboxylase activity is suppressed (62). Succinic acid is present in many leaves and has been identified as the chief component containing labeled carbon when *Chlorella* cells have been exposed to C^{14} in the dark (17). Fumaric acid has been found in leaves of *Taxus baccata* and others (65). Malic acid is perhaps the most widely distributed of all the acids here mentioned (65). Tartaric acid is present in the leaves of *Vitis vinifera* (65). Oxalacetic and α -ketoglutaric acids are constituents of the green parts of red clover (135). Tricarballic acid is contained in the green parts of growing plants and aconitic acid in the leaves of *Helleborus niger* (65). Citric acid is widely distributed in leaves (cf. 134-b), and isocitric acid comprises 18 per cent of the organic solids of very young leaves of *Bryophyllum calycinum* plants (97-a).

The diurnal fluctuation of leaf acidity has been the subject of a number of investigations. The variation in acidity is associated with the fluctuation of carbohydrate and in the opposite direction—low sugar and high acid concentrations in the morning, and high sugar and low acid concentrations in the evening (cf. 119-a). This type of diurnal fluctuation is particularly noticeable in cacti (102) and in crassulacean plants (16-a).

A review of these relationships has been given by Bennet-Clark (16-b) who laid particular emphasis on the increase of sedoheptose in light and its disappearance in darkness in relation to the behavior of acids. He has given evidence for consecutive reactions in the transformation of sedoheptose to malic acid and starch:



Wolf (145-b) has proposed that a close connection exists between the formation of organic acids and the disappearance of fermentable carbohydrates.

Recently, Pucher *et al.* (97-b) have shown in graphic form the inverse relationship between carbohydrates and organic acids—especially malic acid. They have followed the changes in organic acids in *Bryophyllum calycinum* and have summarized their results

as follows: "The change was found to arise largely from alterations in the concentration of malic acid although citric acid shared to a moderate extent. Isocitric acid did not change in concentration in a similar regular progressive manner. Only a trace of oxalic acid was present and no significant change was detected."

That the change in acid concentration is due largely to malic acid was demonstrated by Krebs and Eggleston (70). In *Bryophyllum calycinum*, 83 per cent of the change is due to malic acid, 8 per cent to isocitric acid, and 9 per cent to citric acid; in *Sedum praealtum*, 79 per cent to malic acid, 9 per cent to isocitric acid, and 12 per cent to citric acid.

Particularly noteworthy is the fact that the diurnal fluctuations in the acids of the pineapple leaf are pronounced only in the chlorophyllous portions of the leaf (112).

In rhubarb plants, however, the variation in acidity is in the opposite sense than that reported above. Steinman (124), cited by Vickery *et al.* (134-b) has found "that the titratable acidity . . . increases during the day, . . . which he [Steinman] interpreted as an indication that acids are formed by photosynthesis. The change amounted to 9 per cent of the morning acidity." And Virtanen *et al.* (135) have found an increase in light and decrease in darkness of oxaloacetic acid in clover plants.

The newer experiments with radiocarbon have established the fact that acids are formed in the darkened cell by the incorporation of external CO_2 into carboxyl groups. However, this latter reaction probably accounts for only a small part of the acid production (17).

Ascorbic acid is formed in leaves by illumination and disappears when the leaves are darkened (cf. 136). The amount of ascorbic acid has been positively correlated with the amount of chlorophyll. The ability of chloroplasts to reduce silver nitrate under certain conditions has been ascribed to the ascorbic acid formed by the action of light (142). The relationship of ascorbic acid to photosynthesis is a moot question, for a discussion of which the reader is referred to Rabinowitch (99).

During periods of illumination, the acids which disappear may be converted to carbohydrates. The conversion mechanism is obscure but several hypotheses have been advanced; the carbohydrates may be formed by condensation of the acid molecules, perhaps through some cyclic process (cf. Chap. 19); or by splitting off the carboxyl group as CO_2 and utilizing the CO_2 in photosynthesis; or by a combination of the two paths. Evidence bearing on the transformation has been obtained by Spoehr and by Mayer. Spoehr (119-a) observed that acids disappear from expressed sap when the sap is illuminated and simultaneously CO_2 is evolved. Mayer

(76) showed that plants rich in acids evolve oxygen and increase in starch even though placed in an atmosphere free of CO₂.

In vivo, the splitting of CO₂ from the acids of the leaf may not be a simple photochemical action. The reasons are that it is affected by narcotics and by temperature (16-b), and that the levo-form of malic acid disappears more rapidly than the dextro-form (145-a).

The extreme complexity of the behavior of the acids may be seen by reference to the paper of Vickery *et al.* (134-b).

Protein and Amino Acids

The photosynthetic organs of most plants contain considerable quantities of protein, as the data of Table 3.1 show.

In this connection, it may be significant that green leaves are higher in nitrogen than the variegated forms (34). In leaves of *Ananas bracteatus* the chlorophyllous areas contain more protein

TABLE 3.1
PROTEIN CONTENT OF PHOTOSYNTHETIC ORGANS OF VARIOUS PLANTS

Common Name	Botanical Name	Percentage Protein N, Dry Tissue	Percentage Protein (Protein N x 6.25)	Reference
Liverwort	<i>Lunularia cruciata</i>	2.27	14.19	Lugg, (73b)
Bracken	<i>Pteridium aquilinum</i>	3.69	23.06	Lugg, (73b)
Club-moss	<i>Selaginella</i> sp.	1.82	11.38	Lugg, (73b)
Subterranean clover	<i>Trifolium subterraneum</i>	4.12	25.75	Lugg, (73b)
Cocksfoot grass	<i>Dactylis glomerata</i>	4.50	28.13	Lugg, (73a)
Perennial rye-grass	<i>Lolium perenne</i>	3.38	21.13	Lugg, (73a)
Beet spinach	<i>Beta cicla</i>	3.02	18.88	Lugg, (73a)
Sheep's fescue	<i>Festuca ovina</i>	2.41	15.06	Lugg, (73a)
Barley	<i>Hordeum vulgare</i>	3.95	24.69	Richards and Templeman, (101)

nitrogen than the nonchlorophyllous areas (111). For a fuller account of the proteins in leaves and their behavior under different conditions, the reader is referred to Spoehr and McGee (120), Chibnall (30-c), and Vickery (133).

Is there a diurnal variation in the amino acids and proteins in photosynthetic organs? Chibnall (30-a) has reviewed the earlier work of Suzuki and of Schulze and Schutz on diurnal variation of protein in leaves, and has compared their results with his own. The results of these investigations clearly show a decrease at night in the protein content of leaves. Leaves of runner bean, stored with their petioles in water, showed an increase in nonprotein nitrogen and a corresponding decrease in protein nitrogen whether kept in the light or in the dark, but the change in nonprotein nitrogen was less in the light (30-b). The question of diurnal variation of protein has been considered by Gowentak both from the results obtained by previous workers and from her own experiments (46). She concludes that no regularity in the fluctuation exists but that temperature plays an important role. Higher temperatures favor protein synthesis, lower temperatures favor protein decomposition. Synthesis can take place in the dark under certain favorable conditions when enough nitrogen-containing and nitrogen-free intermediates are present. Gowentak is of the opinion that the influence of light is probably indirect and exerted by the production of the necessary intermediates through the process of photosynthesis.

The influence of light and darkness on the nitrogen metabolism of tobacco leaves has been studied by Vickery and his associates (134-a). Some of the conclusions reached may be quoted: "Digestion of protein with the production of amino acids proceeded rapidly and at approximately the same rate for 72 hours in all experiments. Later, the rate of digestion diminished in leaves cultured in the light, but was maintained in leaves cultured in the dark. . . . Ammonia accumulated in notable amounts toward the end of the experiments in leaves cultured in the dark, but there was only a moderate increase in free ammonia in the leaves in the light. The synthesis of the two amides, asparagine and glutamine, appears to be closely associated with this behavior. Very considerable amounts of asparagine accumulated during dark culture, but the amount of glutamine formed was small. On the other hand, both amides were rapidly synthesized in the light, the quantity of glutamine appreciably exceeding that of asparagine. The total amide synthesis at any point in the light was materially less than the total amide synthesis in the dark. . . .

"In the light, however, photosynthetic reactions provided a bountiful supply of the precursor of glutamine with the result that

this amide was synthesized in rather greater amount than asparagine, and the total amide metabolism was competent to care for all of the ammonia produced in the tissues so that little accumulated. . . . the precursor of glutamine is synthesized during culture in the light, and is, therefore, probably a carbohydrate, or a metabolite of a carbohydrate."

The diurnal variation of nitrogenous components of young leaves of *Bryophyllum calycinum* is very pronounced (97-b). "During the morning and early afternoon, there was a decrease that amounted to about one-third of the protein present in the early morning, but restoration occurred during the night. The curve for soluble nitrogen showed a rise during the day and a fall at night that was symmetrical both in position and magnitude with the change in protein. The increase in soluble nitrogen amounted to about 70 per cent of the early morning value." In excised leaves, the changes during the first twenty-four hours were similar to those occurring in leaves remaining on the plant (97-c).

The protein in detached corn leaves decreased both in light and darkness and residual α -amino acids, asparagine, glutamine, and ammonia accumulated in the leaves. Because of such a decrease, it has been suggested that the synthesis of protein in leaves is under control of hormones from other parts of the plant (132).

A review of the literature on the variation of proteins and amino acids under conditions of light and darkness gives little or no evidence that these substances are direct products of photosynthesis. That the physiological relationships between amino acids, proteins, and carbohydrates are very complex is evident from the experiments of Spoehr and McGee (120) on the respiration of leaves and its acceleration by amino acids.

Lipoidal Material

Lipoids are widely distributed in photosynthetic organs and organisms. Leaves contain a considerable amount of lipid material as the data of Table 3.2 show.

Fats form a considerable percentage of the dry weight of the green alga *Chlorella*. A large portion of the fatty acids of this organism are highly unsaturated (123). Different brown algae have been shown to contain various amounts of lipid material. The differences observed have been ascribed to the habitats of the different species. Those species which live high in the tidal zone contain more lipid than those which are submerged continuously (47-c). The lipid content of some brown algae and the properties of the lipid material are shown in Table 3.3. The very low values obtained in some of these organisms corroborate the low values

obtained by Hoagland (56) in his early survey of the kelps of the Pacific Coast. He found the ether extract of several species of marine algae to be from 0.40 to 1.06 per cent of the dry weight.

As the cells grow old, fat accumulates. Harder and von Witsch (49) found only 5 per cent fat in the diatoms of young cultures but 40 to 50 per cent fat in old cultures.

Spoehr and Milner have followed the degree of reduction ("R-value") of the green alga, *Chlorella pyrenoidosa*, cultured in con-

TABLE 3.2
LIPID CONTENT OF VARIOUS LEAVES

Leaf Source	Nature of Lipoid Material	Percentage of Dry Weight	Reference
Cocksfoot grass (<i>Dactylis glomerata</i>)	Ether extract	2.2	Smith & Chibnall, (116-a)
Rye grass (<i>Lolium perenne</i>)	Ether extract	1.7	Smith & Chibnall, (116-a)
Elephant grass (<i>Pennisetum purpurens</i>)	Ether extract	1.2	Lovern, (72)
Guinea grass (<i>Panicum maximum</i>)	Ether extract	0.95	Lovern, (72)
Cranberry (<i>Vaccinium macrocarpon</i>)	Ether extract	10.57-14.58	Addoms & Mounce, (1)
Rape (<i>Brassica napus</i>)	Fatty acids	1.14	Shorland, (110)

tinuous light for periods up to 63 days. On a scale of "R-values"—carbohydrate 28, protein 42, and fat 67.5—the cultures showed a variation of from 38 to 63. On the assumption that the organisms are composed of carbohydrate, protein, and fat, the compositions of the organisms are calculated for different stages of growth and are tabulated in Table 3.4 (121-c, pp. 101-11). The physical properties of the cells reflect this change in composition, particularly the water content of fresh cells; the water content decreases as the lipoid material increases.

Whether or not there is a diurnal fluctuation of fats in leaves or other photosynthetic organisms is not satisfactorily established by chemical and analytical methods. The rapidity of formation of

oil drops in the light and depletion in the dark has been followed by microscopical observations in photosynthetic organisms by numerous investigators and certainly it is not a very rapid process (9, 81). However, there appears to be some correlation of lipid synthesis with illumination because there is a rapid increase in lipoidal material on illumination of etiolated leaves (81-a, 117-c).

In diatoms, there is an increase in lipoids with increased periods of illumination and there is utilization of lipoids as evidenced by the

TABLE 3.3
THE LIPOID CONTENT AND PROPERTIES OF THE LIPOID MATERIAL FROM CERTAIN
BROWN ALGAE (47c, 108)

	Pet. Eth. Extract*	True Fat*	Unsap- onifi- able*	Fatty Acids in Pet. Eth. Extract†	Iodine Value	Fatty Acid Comp.	
						Solid Acids‡ (%)	Liquid Acids‡ (%)
<i>Pelvetia canaliculata</i> form <i>libera</i>	8.0	6.2	0.62	72.5	107	11.5	78.7
<i>Pelvetia canaliculata</i>	4.9	3.6	0.53	69.9	124
<i>Fucus vesiculosus</i>	2.6	1.9	0.44	71.6	108
<i>Laminaria digitata</i>	0.3	0.16	0.078	49.9	110	17.7	72.2

* The values in the first three columns are percentages of dry weight.

† The figures in the fourth column refer to percentages of the petroleum ether extract itself.

‡ The values in the last two columns are percentages of total fatty acids.

respiratory quotient of diatoms in the dark. These facts suggest that lipoids fluctuate in the organism between periods of light and darkness and are in some way connected with the photosynthetic process. This phase of the photosynthetic problem is very much in need of investigation and clarification.

Phosphorus Compounds

The question whether phosphorus compounds are formed in photosynthetic organs during illumination is a very important one because of the significance it bears to the transfer of energy in the photosynthetic process and the part played by phosphorylations in the transformations of carbohydrates.

Several observers have proved the presence in leaves of phosphorus in organic combination. Glucose phosphate esters occur in *Elodea densa* and wheat and rye seedlings (11) and in linden leaves (12). These compounds have also been observed in beet leaves (25), pea leaves (52-a), tomato plants, box-elder leaves, and shoots (32).

Besides the water-soluble carbohydrate compounds of phosphorus, leaves contain lipoid phosphorus compounds. Chibnall and

TABLE 3.4
CHANGE IN COMPOSITION OF CHLORELLA PYRENOIDOSA
WITH LENGTH OF CULTURE PERIOD (12c)

Culture Period: Days	R-value	Protein (percentage)	Carbohydrate (percentage)	Lipoid (percentage)
14.....	38	58.0	37.5	4.5
21.....	42	50.0	32.3	17.7
28.....	50	28.3	26.2	45.5
42.....	56	15.7	19.0	65.3
63.....	63	8.7	5.7	85.6

his associates have examined leaves of different species for phosphatides (116-b). Cabbage leaves contained no lecithin or kephalin. The main phosphatide was phosphatidic acid. In cocksfoot grass, however, lecithin, kephalin, and phosphatidic acid were all shown to be present. Since phosphatidic acid appears as magnesium salt in the early stages of development of the runner bean and changes to the calcium salt on development of the prophylls, Jordan and Chibnall (63) postulated that the magnesium is utilized for the formation of chlorophyll.

Because of its importance in many phosphorylation reactions it would be of significance to demonstrate the presence of adenosine triphosphate in leaves or other photosynthetic organisms. Indirect evidence of its presence in sugar cane blades has been obtained by the action of specific poisons on sucrose synthesis (50-e) and by increased synthetic efficiency on addition of adenylic acid (50-c). However, other coenzymes containing phosphorus have been reported to be present in leaves. Adenosine-pentose-pyrophosphate has been obtained from oat seedlings (2) which has a ratio of adenine:pentose:phosphorus of 1:1:2. Cocarboxylase occurs in both green and

etiolated barley seedlings (24) and has been shown to be part of the apoenzyme.

The behavior of phosphorus compounds has been little investigated during photosynthesis but there is indication that they are formed by illumination of photosynthetic organisms. Barrenscheen and Pany (12) have shown hexose phosphates to increase on illumination of *Elodea densa* plants, and lipoidal compounds containing phosphorus are formed during the greening of etiolated barley plants (117-c). There is an effect of light and CO_2 on the distribution of organic compounds of phosphorus in *Chlorella* cells (38).

The fact that cocarboxylase occurs in etiolated tissue indicates that its formation in leaves is not dependent on light exclusively.

The enzyme which causes the hydrolysis of phosphate esters occurs in leaves of bean, potato, radish, wheat (61), geranium, rose, lily, and violet (50-e). Its concentration is higher in leaves than in other parts of the plant. Phosphorylase, the enzyme responsible for the condensation of hexose-1-phosphate to more complex carbohydrates, and the reverse process, also occurs in leaves (48-a).

In view of the increasing weight of evidence, it can hardly be doubted that organic compounds of phosphorus are closely associated with the photosynthetic process. Whether they are formed directly in the photosynthetic process or whether they arise by secondary reactions is not clear as yet. However, it is highly probable that they participate in the transformations of the carbohydrates arising from the photosynthetic process and in carboxylation equilibria.

COMPOSITION OF CHLOROPLASTS

Inasmuch as the chloroplast is the seat of photosynthesis, the discovery of what substances exist there may give clues as to what substances are formed as the direct product of photosynthesis. For a long time, it has been known that during photosynthesis in many plants starch grains are built in physical contact with the chloroplast. This has been one principal argument in favor of starch being a direct product of photosynthesis. This argument is not invulnerable; nevertheless, the phenomenon itself speaks for a close association of starch formation with photosynthetic action. However, the presence of starch in isolated chloroplasts is variable. When isolated from leaves, the chloroplasts of *Trifolium pratense* contained no starch, but those isolated from *Onoclea sensibilis* contained 8.4 per cent (86).

Analysis of isolated chloroplasts show them to be very rich in protein. They may contain from about 35 to 45 per cent of the total leaf protein (133). On a moisture-free basis, the chloroplasts of

Trifolium pratense contained 50.1 per cent protein and *Onoclea sensibilis* (86), 31.5 per cent.

Protein from chloroplasts differs qualitatively from cytoplasmic protein. For example, the chloroplastic protein from spinach contains significantly less lysine and more histidine, and the chloroplastic protein from Sudan grass contains considerably more sulfur than cytoplasmic protein (95). It is evident that the chloroplast is capable of synthesizing proteins specifically different from the surrounding cytoplasm. Whether this points to a direct relationship between protein synthesis and photosynthetic activity is a matter of speculation.

A high concentration of lipid is found in the chloroplasts. Menke (79, 80) has found the chloroplasts of spinach to contain about 37 per cent lipoidal material whereas the cytoplasm contains only about 1 per cent. The ether-soluble fraction from the chloroplasts consists of pigments, fatty acids, glycerides, phosphatides, steroids, hydrocarbons, waxy components, carbohydrates, and inorganic components. When separated by solubility in acetone, 79 to 84 per cent is soluble, and from 16 to 21 per cent insoluble. The acetone-soluble material contains from 5.74 to 6.59 per cent glycerine, from 15.91 to 20.92 per cent of nonsaponifiable material of which 11.13 to 14.35 per cent is sterin, and from 45.33 to 55.25 per cent of fatty acids. The fatty acids have an average molecular weight of 275.7 to 285.8, and an iodine number of 184.7 to 210.0. The lipid contained about 2 to 7 per cent phosphatide and 15 to 17 per cent crude wax. However, the work of Neish (86) makes it clear that the quantity of lipid varies considerably in chloroplast material; in *Trifolium pratense* the lipids constituted 21.75 per cent and in *Onoclea sensibilis* only 7.21 per cent. The composition of the lipoid fractions of chloroplasts from cabbage and cocksfoot grass are given by Chibnall (30-c).

INCREASE IN ORGANIC MATTER RELATIVE TO INCREASE IN CARBON

Just as the photosynthetic quotient gives circumstantial evidence of the over-all nature of the organic products formed in photosynthesis, so does the increase in weight of leaves relative to the amount of CO_2 absorbed.

Krascheninnikov (69) examined the leaves of five species of plants by the half-leaf method and found that the increase of weight relative to CO_2 absorbed was on the average 0.654. This value closely approximates the theoretical ratio for a disaccharide, 0.648.

Forty-two years later, Smith (117-a) examined the increase in dry weight of sunflower leaves by the half-leaf method as related to the uptake of CO_2 and to the increase in carbon as determined by

elementary analysis. As the result of six determinations the increase in carbon, as determined by absorption of CO_2 , was 40.9 ± 0.9 per cent of the increase in dry weight. The increase in carbon as determined by elementary analysis was 41.8 ± 1.1 per cent of the increase in dry weight. The average of the two methods was 41.4 ± 0.6 per cent. This agrees closely with the carbon percentage in a disaccharide, 42.1 per cent, and is indirect evidence that the product of photosynthesis is carbohydrate.

Österlind (91) has compared the dry-weight accumulation of a green alga, *Scenedesmus quadricauda*, with the amount of carbon absorbed as CO_2 in photosynthesis. His calculations show that carbon comprises 70 per cent of the dry weight accumulated, which indicates "that fats constitute a great part of it." This contrasts greatly with the results on higher plants.

HEAT OF COMBUSTION OF ORGANIC MATTER NEWLY FORMED IN PHOTOSYNTHESIS

Another method used to gain information concerning the over-all products of photosynthesis is that of heat of combustion, i.e., the increase in the heat of combustion of a given area of leaf brought about by a definite amount of photosynthesis.

Krascheninnikov (69), the first to use this method, found the average increase in heat of combustion per gram of CO_2 absorbed to be 2,728 gm. cal. The values in gm. cal. for the conversion of 1 gm. of CO_2 to various substances are as follows: sucrose, 2,556; cellulose, 2,568; protein, 2,966; and fat, 3,328. The increase in heat of combustion for 1 gm. increase in dry weight was 4,373 gm. cal., whereas had the following substances been formed exclusively the values would have been: glucose, 3,736; sucrose, 3,943; starch, 4,179; cellulose, 4,181; protein, 5,620; and fat, 9,500 gm. cal. From these data, the products of photosynthesis appear to be above carbohydrate in energy but considerably below protein and fat.

Puriewitsch (98) also used this method and obtained the results shown in Table 3.5.

The values obtained by heat of combustion differ for the different plants. Except for the case of sunflower they are higher than would correspond to a carbohydrate. This exception may be significant since sunflower is a well-recognized carbohydrate synthesizer. In most instances, the evidence from heat of combustion points to a part of the photosynthate being something else than carbohydrate.

SPECIFIC ORGANIC SUBSTANCES FORMED RELATIVE TO CO_2 ABSORBED

By comparing the amount of carbon absorbed as CO_2 with the amount appearing in new-formed organic substances, a carbon balance can be obtained. From this a quantitative estimate can be made

of the products formed in the up-grade, energy-storing process. The shorter the time of illumination and the shorter the period between illumination and the preparation of the materials for analysis, the more reliable the results should be. Although this method has obvious disadvantages, no other way was available for such an inquiry before the development of methods which use isotopic carbon. But tracer methods also have their limitations.

TABLE 3.5
HEAT OF COMBUSTION OF MATERIAL NEWLY FORMED IN PHOTOSYNTHESIS (198)

	Average Increase in Dry Weight per Sq. Cm. of Leaf Surface	Average Increase in Heat of Combustion	
		Per Sq. Cm. of Leaf Surface	Per Gm. of Dry Weight
	gm.	gm. cal.	gm. cal.
<i>Polygonum sacchalinense</i>	0.00085	3.980	4682
<i>Acer platanoides</i>	0.000725	3.170	4372
<i>Saxifraga cordiflora</i>	0.0008	3.450	4313
<i>Helianthus annuus</i>	0.0015	5.977	3985

To get a reasonably complete picture of the transformation brought about by photosynthesis, the use of both methods is necessary.

The earliest attempt to determine a balance between carbon absorbed and carbon appearing in known compounds was made by Saposchnikov (109-b). In three experiments on leaves of *Helianthus annuus*, the carbohydrates formed, reckoned as glucose, corresponded to 67.6, 87.1, and 63.8 per cent of the theoretical amount calculated from the amount of CO₂ absorbed. The deficit he attributed to protein formation, without offering any experimental evidence for this assumption.

Later, Krascheninnikov (69) determined the increase in carbohydrate in relation to the CO₂ absorbed. For leaves of five different plants he obtained the following ratios: bamboo, 0.45; cherry laurel, 0.31; sugar cane, 0.50; linden, 0.56; and tobacco, 0.37. The theoretical stoichiometric ratio is 0.682. On the average only 64.2 per cent of the required carbohydrate was recovered by analysis. This experiment also shows a discrepancy between the amount of carbohydrate determined by chemical methods and the theoretical amount calculated from the amount of CO₂ absorbed.

More recently the relation between the amount of CO₂ absorbed

and the organic products of photosynthesis in wheat plants has been studied by Burström (26-a). Detached leaves were used, thereby avoiding transport of material from the leaf. The assimilation of mature and young wheat leaves containing various quantities of nitrate were examined at low and high light intensities.

Mature leaves of low nitrate content illuminated with low light intensities (9,000 lux) converted all the CO_2 absorbed into sugars—hexoses and sucrose. When the nitrate content was raised, the CO_2 absorption was increased to the extent necessary to convert the nitrogen to protein. The conclusion was that “only two products appear, sugar and protein, or their precursors. They must both be regarded as primary assimilates, because nitrate neither reacts with stored sugar in darkness nor in light without CO_2 supply. It is also a noticeable fact that the formation of sugar is rather independent of protein synthesis . . . the two kinds of assimilates simply add themselves, so that the total assimilation of CO_2 considerably increases in the presence of nitrate.” At high light intensities (35,000 lux) the sugar and protein are not formed independently and addition of nitrate does not increase significantly the uptake of CO_2 . The formation of sugar is apparently diminished by the formation of protein.

In vigorously growing leaves the sugar produced does not account for all of the CO_2 taken up in photosynthesis. In comparison to the nitrate assimilated, much more carbon is fixed in compounds other than sugar than corresponds to protein. These nitrogen-free, non-sugar compounds are formed independently of or at the expense of sugar. Their nature and their mode of formation are still undetermined.

A summary of these results is given in Table 3.6.

The work of Burström leaves little doubt that nitrate assimilation is connected in some way with CO_2 photosynthesis (26-b). The mechanism of the reaction is by no means settled but the suggestion is made that an intermediate product of carbon assimilation reacts with an intermediate product of nitrate reduction to form protein.

In a similar investigation, Smith (117-a, -b) showed that under certain circumstances all of the CO_2 absorbed by detached sunflower leaves could be accounted for by increase in carbohydrate. The values were determined for various periods of time and for different classes of carbohydrates in order to find out what sequence the formation of the different carbohydrates followed. The results of this investigation are shown in Figure 3.3.

These experiments were of relatively short duration in order to avoid as far as possible transformation of the substances formed during photosynthesis. For the four periods of photosynthesis used,

the average times of illumination for the replicate experiments were 27, 58, 101, and 146 minutes.

As the periods of illumination increased, all of the constituents increased in amount except the "residue." The monosaccharides increased in amount more than in direct proportion to the CO_2 absorbed. Aldoses and ketoses appeared in approximately equal amounts—ketoses comprising about 44 per cent of the increase in monosaccharides. Sucrose increased, but less than proportional to

TABLE 3.6
THE ASSIMILATION OF CO_2 AND NITRATE (26A)

Leaf	Nitrate Level	Change in *				C:N	Intensity Lux
		CO_2	Sugar	Other Products	Nitrate		
Mature.....	Low	0.061	0.059	0.002	-0.002	9,000
Mature.....	Normal	0.097	0.053	0.044	-0.049	5.4	9,000
Growing.....	Low	0.063	0.042	0.021	-0.005	9,000
Growing.....	Normal	0.071	0.003	0.068	-0.024	17.0	9,000
Mature.....	Low	0.244	0.242	0.002	0.002	35,000
Mature.....	Normal	0.266	0.231	0.035	-0.055	3.8	35,000
Growing.....	Low	0.203	0.144	0.059	-0.005	35,000
Growing.....	Normal	0.221	0.084	0.137	-0.045	18.3	35,000

* The changes within the leaves are for 1 gm. of fresh leaf material and 24 hours. The figures for CO_2 and sugar are given in equivalents of hexose.

the CO_2 absorbed. The sums of monosaccharides plus sucrose increased in direct proportion to the amount of CO_2 absorbed. The starch also increased in direct proportion to the CO_2 absorbed. Minor constituents, designated "sugar not identified" and "polysaccharide not identified," increased also to some extent but their true relations to the amount of CO_2 absorbed could not be definitely determined.

The behavior of the "residue" fraction is remarkable. It was increased by addition of CO_2 in the dark and decreased progressively by illumination, and as more CO_2 was assimilated. Whether it functions as an intermediary in photosynthesis is unknown at the present time.

The total carbohydrates, determined by volumetric procedures, increased in almost direct proportion to the amount of CO_2 absorbed during illumination. The increase in carbohydrates accounted for nearly all of the carbon assimilated as CO_2 — 98.7 ± 2.1 per cent in experiments carried out at 10.0°C . and as high as 96.7 ± 2.7 per cent at 20.0° .

From the results obtained it was concluded that "starch and sucrose formed concurrently in side-by-side reactions, and that sucrose is subsequently inverted to monosaccharides. The possibility exists that sucrose and starch arise from a common precursor."

The ratio of the different carbohydrate fractions to the CO_2 absorbed varied with the temperature at which the experiment was performed. The percentages of the carbon absorbed at 10° and 20° which were recovered in the principal carbohydrate fractions were as follows: monosaccharide, 7.1 and 10.0; sucrose, 71.0 and 51.8; and starch, 16.1 and 25.5 (117-a).

In all of the experiments reported to this point, the quantitative relations between the amount of CO_2 absorbed and the increase in amount of the various constituents of photosynthesizing tissue has been used as the criterion to judge what substances are formed from the CO_2 assimilated and in what proportion. That the substances which increased were actually formed from the CO_2 absorbed was impossible to determine. For such determinations isotopic carbon has proved to be a boon.

The first experiments taking advantage of this new tool were performed by Ruben, Hassid, and Kamen (106) who used the short-lived radiocarbon, C^{11} . A summary of their results on barley plants is included in this volume in a paper by Kamen (Chap. 18). In their early experiments with barley leaves, they recovered from 3 to 5 per cent of the photosynthesized C^{11} in the simple sugars and from 11 to 25 per cent in the total water-soluble carbohydrates. Some radiocarbon was detected in water-insoluble material and in chlorophyll. In experiments with *Chlorella*, illumination for from one to five minutes produced organic substances containing radiocarbon which were not identified (105). The material formed was reported to be nearly homogeneous and to have a molecular weight of about 1,000 (103, 104)—a result which appears to be doubtful in the light of more recent work.

The distribution of radiocarbon, C^{14} , has been measured in young barley plants after a period of one hour's photosynthesis in radioactive CO_2 and about two hour's respiration (7). In these experiments one set of plants with roots and another set without

roots were used. Although differences in distribution of the radio-carbon were noted between the two sets of plants, the general picture was the same. The most striking effect was the widespread distribution of the C^{14} among the various classes of organic compounds. As might be expected, the sugars contained the greatest amount of C^{14} , acids the next largest amount, and an appreciable quantity was present in the amino acid fraction. The cell-wall materials, cellulose and lignin, also contained a significant amount of the labeled carbon. These results differ in some respects from those obtained by Ruben and his co-workers; however, the results agree in pointing to the wide distribution of newly photosynthesized carbon among the different classes of compounds.

When bean plants, three weeks old, were exposed to CO_2 containing excess C^{13} and illuminated for a half hour, the sucrose fraction contained a higher concentration and far more C^{13} than any of the other fractions. "The results indicate that CO_2 is converted most rapidly to sucrose." After three days of illumination the C^{13} was approximately equally concentrated in the glucose, sucrose, dextrin, and starch fractions (146).

The work of Frenkel (43) has thrown light on the interesting question of the site of photosynthesis and the nature of the products formed. After exposure of *Nitella* cells to radioactive CO_2 in the light for 25 minutes, the chloroplastic material contained about 80 per cent of the radioactivity and this activity could not be removed by washing with 0.5 M glucose solution. The product, therefore, appears to be either little soluble or little diffusible in water solution.

NUTRITION OF ALBINO PLANTS

"The theory that carbohydrate is the only product of photosynthesis and that the entire organic nutrition of the plant can ultimately be satisfied from this material can, in a measure at least, be subjected to experimental test . . . By entirely excluding photosynthesis a test could be made to determine to what extent a higher plant can be brought to full development by means of artificial nutrition exclusively with a carbohydrate . . . means are available for such an experiment: the culturing through artificial nutrition of . . . albino plants. These plants are devoid of the capacity for photosynthesis. . . . albino maize plants can be kept alive for several months by artificial organic nutrition with sucrose. These plants showed a definite increase in dry weight above that of the seeds from which they sprang. They produced the same number of leaves as normal green plants and also staminate and pistillate inflorescences. The albino leaves formed starch in the dark when infiltrated

with solutions of sucrose and dextrose, but, in contradistinction to green leaves, not with solutions of glycerine or sorbitol" (Spoehr, 119-d). Albino maize plants, fed on sucrose, contained about twice as much uronic acid per unit dry weight as normal plants grown in the light (122).

Albino plants grown under these conditions do not attain the vigor of normal plants grown in the light. Other nutritional factors must be formed by the normal plant which are necessary for normal development. But the amazing fact is that a higher plant can live and grow for so long a time when nourished with but a single pure organic substance—sucrose.

SUBSTANCES FORMED IN RECIPROCAL GRAFTS

Whether or not a specific substance in a leaf is the product of photosynthesis may be tested by the method of reciprocal grafts. By grafting two types of plants, one of which contains a certain substance in its leaves while the other does not, it can sometimes be determined whether or not this substance is a product of photosynthesis. In the two types of graft the top of one plant is grafted to the roots of the other, and *vice versa*. If the substance is formed in the top of the first plant when it is attached to its own roots but not when it is attached to the root of the second plant, and when the top of the second plant forms the substance when attached to the roots of the first plant but not when attached to its own roots, it is fairly certain that the substance in question is not a direct product of photosynthesis.

As an example of this method *Datura* and tomato plants have been grafted reciprocally to determine whether the alkaloid, hyoscyamine, normally produced in *Datura* plants, is formed in the leaves or in the roots. The alkaloid was not found in *Datura* scions grown on tomato roots, but tomato scions grown on *Datura* roots yielded 0.12 per cent of the dry weight of the leaves as a crude alkaloidal complex. The conclusion is, therefore, that the alkaloid is formed in the roots and cannot be a direct product of photosynthesis (93).

"FIRST PRODUCT" OF PHOTOSYNTHESIS

ORGANIC ACIDS

One of the first hypotheses concerning the mechanism of photosynthesis proposed that organic acids are the primary products formed by photosynthetic reduction of CO_2 (119-c, pp. 259 ff.; 99, pp. 262 ff.). As knowledge of the process increased, however, this hypothesis became difficult to accept. In those plants in which the acids are most abundant the acid content decreased on illumination

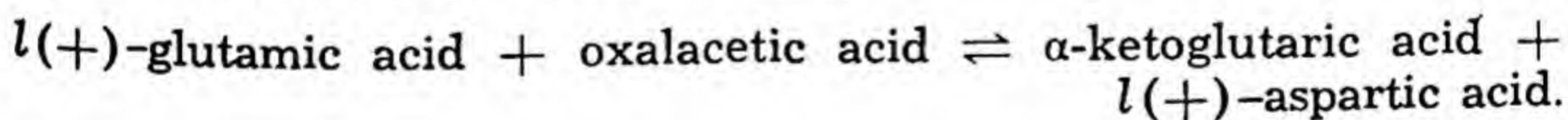
and increased in the dark—a fact which was at variance with the photosynthetic origin of the acids.

Further examination showed that a reciprocal relation exists between the acids and carbohydrates. In the light, acids disappear and carbohydrates increase, and in the dark carbohydrates disappear and acids increase⁴ (cf. p. 66).

One present-day concept of the mechanism of photosynthesis links the formation of carbohydrates with the disappearance of acids through a cyclic process which is driven primarily by reducing agents resulting from photochemical action (cf. Chap. 19). Such a cycle further provides for the formation of acids by absorption of CO₂ from the surroundings, thereby providing the acid intermediates necessary to the continuous production of organic matter—notably carbohydrates—in the photosynthetic process (17, 106, 118). The older vague ideas relating respiration and photosynthesis (cf. 120) can be given more exact formulation by such a cycle as that which has been proposed by Benson and Calvin (17), and shown on page 399 of this volume.

The formation of chlorophyll from protochlorophyll in illuminated etiolated barley seedlings is a mole-for-mole transformation (Chap. 9). On the basis of chemical structures proposed by Fischer (41, 42), this reaction is a hydrogenation. In mature barley leaves taken from the field, these two pigments co-exist (66). These facts suggest that the chlorophyll system itself may be the source of the hydrogen necessary to drive a cycle of the kind proposed by Benson and Calvin.

The synthesis of amino acids and proteins may also be tied to the metabolism of the keto-acids. Systems in plants have been isolated in which the amino group in the amino acids can be transferred to keto-acids (20, 125, 137). For example the following equilibrium has been shown to exist:



A whole series of such transaminations has been found in biological material and may account for the formation of the proteins in plants (cf. 20, pp. 22, 36).

FORMALDEHYDE

A "first product" of photosynthesis which has been the subject of much discussion is formaldehyde. The hypothesis of Baeyer, in which formaldehyde is assumed to be produced by the direct reduction of CO₂ and then polymerized to sugars, has been a favored hypothesis largely because of its simplicity and its adaptability.

One reason for giving this theory little credence is the failure to detect formaldehyde in photosynthesizing organisms. Two attempts have recently been made to detect formaldehyde in photosynthesizing organisms by novel techniques. The results of these experiments have led to opposite conclusions. It has been reported that by use of the magneto-optic method a small quantity of formaldehyde, 3.6 parts in 10^{12} , has been detected in *Chlorella* and that the quantity increased to 5 parts in 10^{10} on illumination of the alga (113, 114). However, by the use of radiocarbon no evidence for the presence of formaldehyde could be obtained in *Chlorella* (105). In view of the doubtful existence of formaldehyde in significant quantity in photosynthesizing tissue, it appears to be unlikely that formaldehyde is the first product formed in photosynthesis. Also, the pattern of the distribution of labeled carbon in the sugars newly formed in photosynthesis makes the formaldehyde hypothesis very improbable.

CARBOHYDRATES

Glucose, fructose, sucrose, and starch are the carbohydrates which have received the most attention as the first products of photosynthesis. The evidence from the older investigations for the primary synthesis of these compounds has been exhaustively reviewed by Priestly (96), Stiles (126), Spoehr (119-c), Hartt (50-a), and by Rabinowitch (99). The older evidence for and against these being the first products may be stated briefly as follows—because starch appears so quickly on illumination of starch-free leaves and because it shows so great variation in amount between periods of light and darkness, it has been held to be one of the primary products formed in photosynthesis. However, it is doubtful that starch is universally a primary product because it is absent in certain photosynthetic organs and because it is structurally so complex. Sucrose may be the first product because it is so widely distributed in photosynthetic organisms and because it fluctuates so greatly between day and night. On the other hand, in some cases, it fails to increase immediately on illumination of the plant, and it also is composed of simpler sugars; consequently it is not likely to be the "first product." The hexoses have been favored as the first product largely because they are the simplest sugars to be widely distributed in plants and to undergo diurnal variation. On the contrary they have not been favored as the first product because they show less diurnal variation than sucrose and starch and sometimes appear to result from hydrolysis of sucrose.

As between glucose and fructose being the first hexose formed, an experiment of Hartt (50-d) is of particular significance. "Detached blades [sugar cane] supplied with water in the light accumulated

glucose but not fructose, when given either arsenite or selenite. Since blades known to be supplied with glucose accumulate glucose, and blades known to be supplied with fructose accumulate fructose, in the presence of arsenite or selenite, the results obtained with blades in water in the light in the presence of these poisons, constitute strong evidence that the first sugar formed in photosynthesis is glucose alone." However, it is difficult to reconcile the fact that some algae contain no soluble sugars, even after vigorous photosynthesis, with the hypothesis that glucose is the only primary product of photosynthesis.

In the more recent experiments relating the formation of carbohydrates to the CO_2 absorbed during photosynthesis, indication has been obtained that precursors are formed which are rapidly and simultaneously converted into more stable products.

The experiments of Smith (117-a, -b) show that the greatest part of the CO_2 absorbed by sunflower leaves during photosynthesis can be accounted for by the increases in the sucrose and the starch fractions. These carbohydrates begin to accumulate immediately upon illumination of the leaf and increase concurrently and in direct proportion to the CO_2 absorbed (cf. Fig. 3.3). This behavior suggests that both carbohydrates are formed from a common precursor, the nature of which is still unknown.

That these carbohydrates are not direct products of photochemical action but depend on chemical transformation of precursors is supported by the fact that the proportions of the carbon absorbed as CO_2 and recovered in sucrose and in starch vary with the temperature. This fact is inconsistent with direct photochemical formation of these substances.

Burström followed the simultaneous uptake of CO_2 and disappearance of nitrate in illuminated mature wheat leaves. His experiments gave evidence that precursors were formed which could be utilized for production of carbohydrate exclusively when nitrate was lacking, and for the simultaneous production of both carbohydrate and protein when nitrate was present. He concluded that, "*In photosynthesis of mature wheat leaves only two products appear, sugar and protein, or their precursors. They must both be regarded as primary assimilates because nitrate neither reacts with stored sugar in darkness, nor in light without CO_2 supply*" (26-a).

With young leaves the results were not so clear-cut. The metabolic reactions attendant on vigorous growth obscure the photosynthetic reactions. However, "the same primary mechanism must exist in both cases . . . but because the leaves are histologically capable of growth, the protein production provokes a building of new cells, including also a formation of N-free building stones of

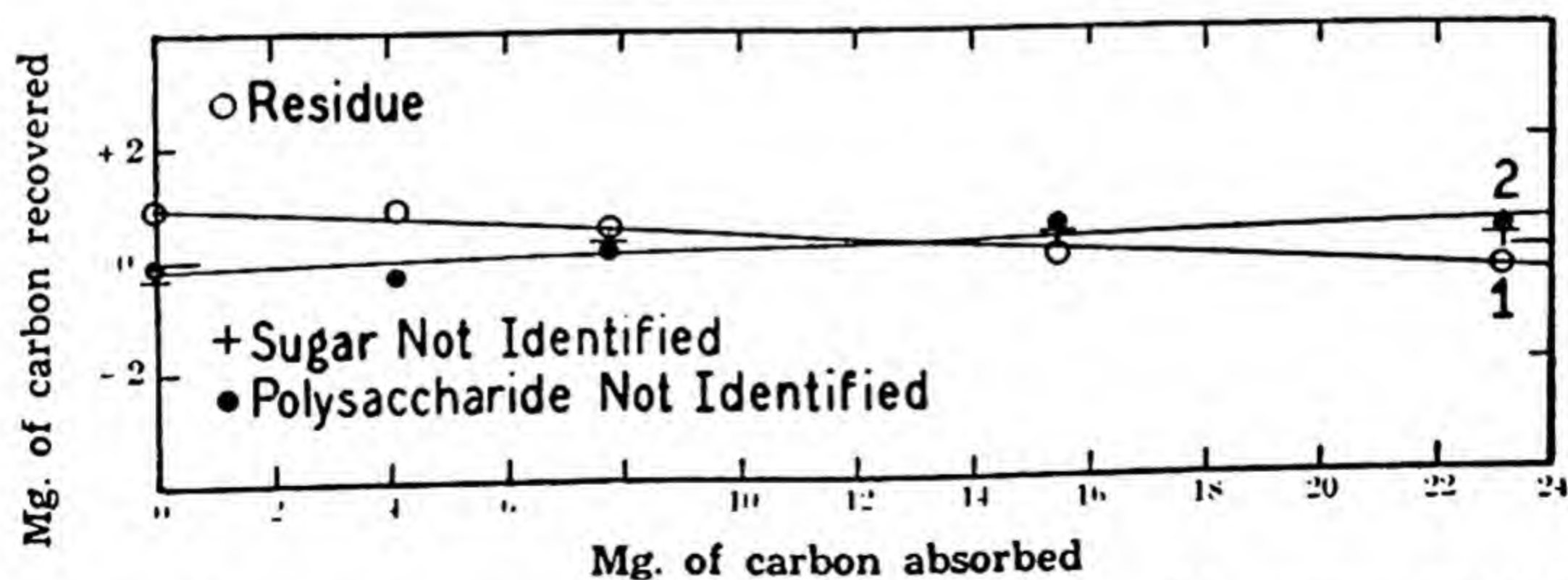
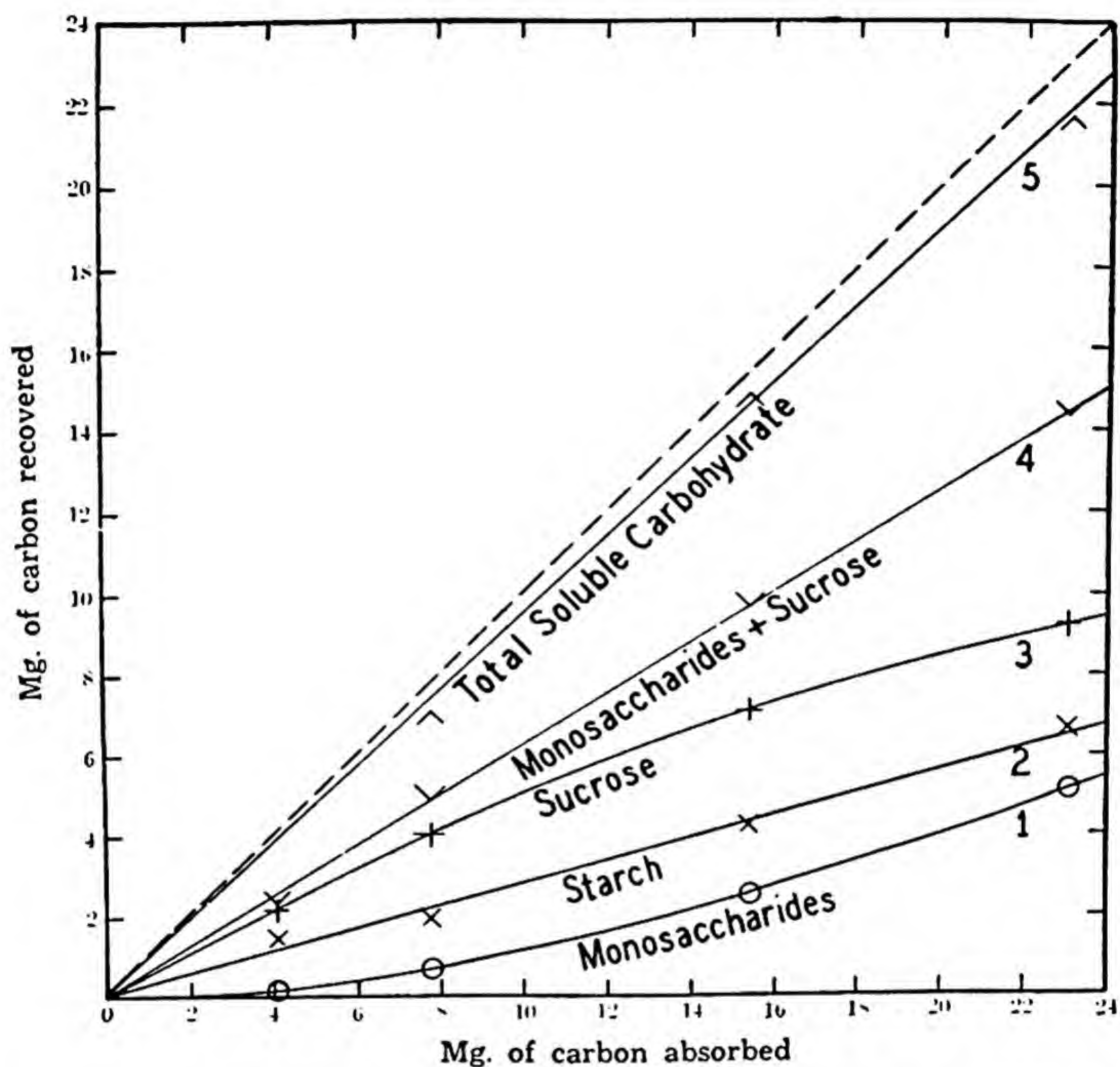


FIG. 3.3—Correlation of the amount of carbon recovered in various carbohydrate fractions with the amount of carbon absorbed in photosynthesis by sunflower leaves. Temperature: 20.0°C. (Smith [117b]).

protoplasm and cell walls. These arise at the expense of, or instead of, sugar."

According to Ruben and his associates, the first detectable product formed by *Chlorella pyrenoidosa* has a molecular weight of approximately 1,000 (103, 104).

Bean plants exposed to $C^{13}O_2$ and illuminated for a half hour contained a higher concentration and considerably more C^{13} in the sucrose fraction than in the glucose, dextrin, or starch fractions. When illuminated for three days, the C^{13} was about equally concentrated in all fractions. Preliminary experiments indicate that the C^{13} is more concentrated in certain positions of the sugar molecules than in others (146). From these results it appears that CO_2 is converted most rapidly to sucrose and that it is doubtful if free glucose is an intermediate in the process.

Glucose obtained from barley plants after about one hour's illumination showed the following distribution of relative quantities of labeled carbon in the different positions: carbons 1, 6, 0.68; carbons 2, 5, 1.00; and carbons 3, 4, 2.61 (6). Such a distribution is difficult to reconcile with either a serial or a random joining of carbon atoms. Rather it implies a union of two preformed moieties produced by a cyclic process such as the one described in Chapter 19 of this volume. This isotopic distribution is perhaps as convincing evidence as any yet available that the sugars arise from smaller molecules through metabolic processes.

If photosynthesis proceeds in some such manner as described by the proposed cyclic process, then it would be difficult to designate which compound is the "first product." What with addition of CO_2 to form acids, the reduction of acids with hydrogen made available directly or indirectly by photochemical action, and phosphorylations to accelerate condensations, the "first product" in the sense in which it has usually been used may cease to have meaning.

TRANSFORMATIONS AND INTERCONVERSIONS

One reason why it is difficult to determine what sugar is first formed by the photosynthetic process is that the sugars in the leaf are transformed into each other. This has been well established by numerous feeding experiments on leaves and algae, summaries of which have already been published (88, 90, 99, 119-c). In the leaf, glucose, fructose, sucrose, and starch—the carbohydrates most commonly encountered—are interconvertible. Therefore, no matter which of these is first formed, the others will soon appear. Even the sugar alcohols—glycerine, mannitol, sorbitol (90, 119-c), and in one instance ribitol (129)—form starch when fed to certain leaves.

This ability to interconvert the sugars is not limited to green leaves and the green portions of variegated leaves. Such transformations occur also in etiolated leaves (77), albino leaves (50-b, 119-d), and in the colorless portion of variegated leaves (109). Albino leaves and the colorless portions of variegated leaves differ from green leaves in not converting the sugar alcohols into starch. It is clear that transformations are not limited to photosynthetic tissue.

Even those sugars which are not found in the free state in leaves are converted into sucrose and other carbohydrates. For example, the triose, glyceraldehyde, as well as mannose and galactose, produces sucrose when fed to barley leaves (77). The reverse transformation, if it occurs at all, produces only very small quantities of these sugars in the free state.

Although galactose and mannose are not found in the free state, the complex carbohydrates formed from them constitute a large part of the cell-wall material in many plants—particularly in the algae. Because of this it has been proposed that these plants have a carbohydrate metabolism based on galactose or mannose systems rather than on the glucose system prevalent in the higher plants (18, 51).

Our understanding of the formation of the complex carbohydrates has been greatly increased through the discovery of the formation *in vitro* of the complex carbohydrates from glucose-1-phosphate. Since the initial work of Hanes (48) on the formation of starch from glucose-1-phosphate, a number of disaccharides have been synthesized by reacting glucose-1-phosphate with a number of monoses (53).

The phosphorylase responsible for the formation of starch from glucose-1-phosphate has been found in leaves (48-a). It is possible, therefore, that the same or a like mechanism for the formation of complex carbohydrates is operative in leaves, although it has not been demonstrated experimentally.

However, it is not necessary to have phosphosugars in order to build up new complex carbohydrates. If in place of glucose-1-phosphate, a glucoside such as sucrose is treated with another monosaccharide in the presence of an enzyme (transglucosidase) a transaccharide in the presence of an enzyme (transglucosidase) a transaccharide of the glucose moiety can be accomplished and a new disaccharide formed. Examples of this reaction are the following (36):

$$\text{glucose-1-fructoside} + \text{sorbose} \rightleftharpoons \text{glucose-1-sorbose} + \text{fructose},$$

$$\text{and glucose-1-ketoxylside} + \text{fructose} \rightleftharpoons \text{glucose-1-fructoside} + \text{ketoxylose}.$$

The mechanism of the formation of the hemicelluloses and mucilages in leaves is a matter of speculation. The scheme proposed

assumes the oxidation of the primary alcohol groups in the hexosans to form carboxyl groups (27-b, 119-b, 122). By the decarboxylation of the uronide, pentosans may be formed. However, exception to this mechanism for the formation of pentosans and uronides has been taken by Hirst (55) because the structural design of the pentosans is different from that of the hexosans from which they apparently would be derived and with which they are associated in nature.

CONCLUSION

From this short survey, it may be seen how varied the materials are which are formed in photosynthetic organs and organisms. It is a question whether these materials arise from a common photosynthetic substance in all organisms, or even within a single organ, or whether they arise from different kinds of photosynthate. On the one hand, feeding experiments have demonstrated that a variety of substances may be produced from a single substance—a fact which eliminates the necessity for assuming the direct photosynthetic production of a variety of substances. On the other hand, the more recent experiments indicate that diverse substances—various carbohydrates, proteins, and acids—arise simultaneously by photosynthetic action.

How such diverse products may arise can be seen from hypothetical cyclic schemes proposed for combining respiratory with photosynthetic action. Such schemes give rather definite expression to the older vague notions of the dependence of photosynthetic action on cellular metabolism. Inasmuch as the metabolic processes may vary in different organisms it would not be surprising to find the products of photosynthesis to vary in different organisms. In these cyclic schemes, it would be difficult to determine which constituent is the "first product."

The distinction is clear-cut between two extreme concepts of photosynthesis: one in which CO_2 and water are combined directly to form carbohydrate and liberate O_2 through some photosensitized reaction; and the other in which CO_2 is incorporated into organic combination and is ultimately transformed into a variety of substances by a series of metabolic steps. These step-wise reactions are driven forward by the uptake of hydrogen ultimately derived from the photochemical decomposition of water. Current opinion largely favors the second of these concepts. The detailed examination of substances involved in the assimilation of CO_2 by photosynthetic action under different conditions and in a variety of organisms will eventually reveal how similar the photosynthetic process is throughout nature.

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4.

Diffusion Through Multiperforate Septa

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PHOTOSYNTHESIS in vascular plants may be limited, among other factors, by the rate at which CO_2 diffuses into the leaf. Carbon dioxide is soluble in the cutin layer which covers most leaves, but at normal concentrations absorption is essentially confined to diffusion through stomata (1). Stomata have a maximum average diameter of the order of 10μ and a frequency of the order of $10,000/\text{cm}^2$ (cf. Table 4.2). The open surface of the stomata may thus approach 1 per cent of the area of the leaf, or they may, as in maize, be so consistently and tightly closed that their area is unmeasurable. An average open area on the order of 0.1 per cent is probably typical.

Carbon dioxide absorption and water loss by leaves are thus dependent upon diffusion through microscopic pores in a multiperforate membrane. Measurements under favorable conditions show that leaves may absorb CO_2 through these stomata at a rate approaching that of a free caustic soda solution, or lose water at the rate of a free water surface. These astonishingly high diffusion rates have been explained by the application of Stefan's diameter law (1). Stefan (8) derived an expression for evaporation from a circular or elliptical plane surface by using an analogous equation from electrostatic theory. His derivation indicated that steady state evaporation from such a surface, if air were perfectly quiet, would be proportional to the diameter of the surface rather than to the area. Stefan visualized "uniform pressure surfaces," also called diffusion shells, extending outward from the plane. These would be ellipsoids, and he says that the stream lines of diffusion are perpendicular to this family of ellipsoids.

One form of Stefan's expression for total evaporation (V_1) from a circular plane is as follows:

$$V_1 = k(a - \sqrt{a^2 - r^2}) \log \frac{P - p_0}{P - p_1} \quad (1)$$

where

a = distance from center of a hyperbola to focus

r = radius of circular plane

k = a proportionality constant

P = pressure of surrounding air

p_0 = vapor pressure at a great distance

p_1 = vapor pressure at the surface of the plane.

It is important to note that this equation yields diameter proportionality only under the special condition that a equals r . The family of hyperbolas which represent the stream lines are subject to this condition; consequently, the uniform pressure ellipsoids with which these stream lines are associated must represent a special group. The special nature of these ellipsoids can best be illustrated by referring to Figure 4.1.

The family of ellipsoids which meets Stefan's conditions—that the distance between the center and focus be constant and equal to the radius of the plane—is peculiar in that the ellipsoids rapidly approach a spherical shape with increasing size. If diffusion shells conform to this shape, then the diffusion shell which has a long axis of twice the diameter of a pore will be distinctly flattened, its short axis being only 1.73 diameters. But the diffusion shell whose long axis is five diameters is practically spherical, its short axis being 4.9 diameters.

It is the spherical shape of diffusion shells which causes the diameter proportionality of small-pore diffusion. This concept is inherent in Stefan's analysis but it has not received adequate recognition. When the diffusion conditions are such that spherical shape is not attained or closely approached by the diffusion shells, diffusion rates will not show diameter proportionality. If the diffusion shells remain ellipsoidal, the diffusion rates will lie somewhere between diameter and area proportionality. Strongly flattened ellipsoidal shells, of course, will yield diffusion rates closely approaching area proportionality.

The obvious factor which would prevent attainment of spherical diffusion shells is air turbulence. Thus Sierp and Noack (7) found a shift from diameter proportionality to area proportionality occurring as wind velocity over an evaporating surface increased. The evaporation equation of Thomas and Ferguson (9), $E = Ka^n$, where a is diameter of evaporating surface and n varies between 1 and 2, applies throughout the range of ellipsoidal diffusion shells, the value of n approaching 2 as the ellipsoids remain more strongly flattened.

It also becomes evident why small pores show diameter proportionality experimentally when larger pores do not. Even the quietest room contains air currents, and these will prevent the formation of the large spherical surfaces required by large pores, but will be relatively less important as pore diameter decreases.

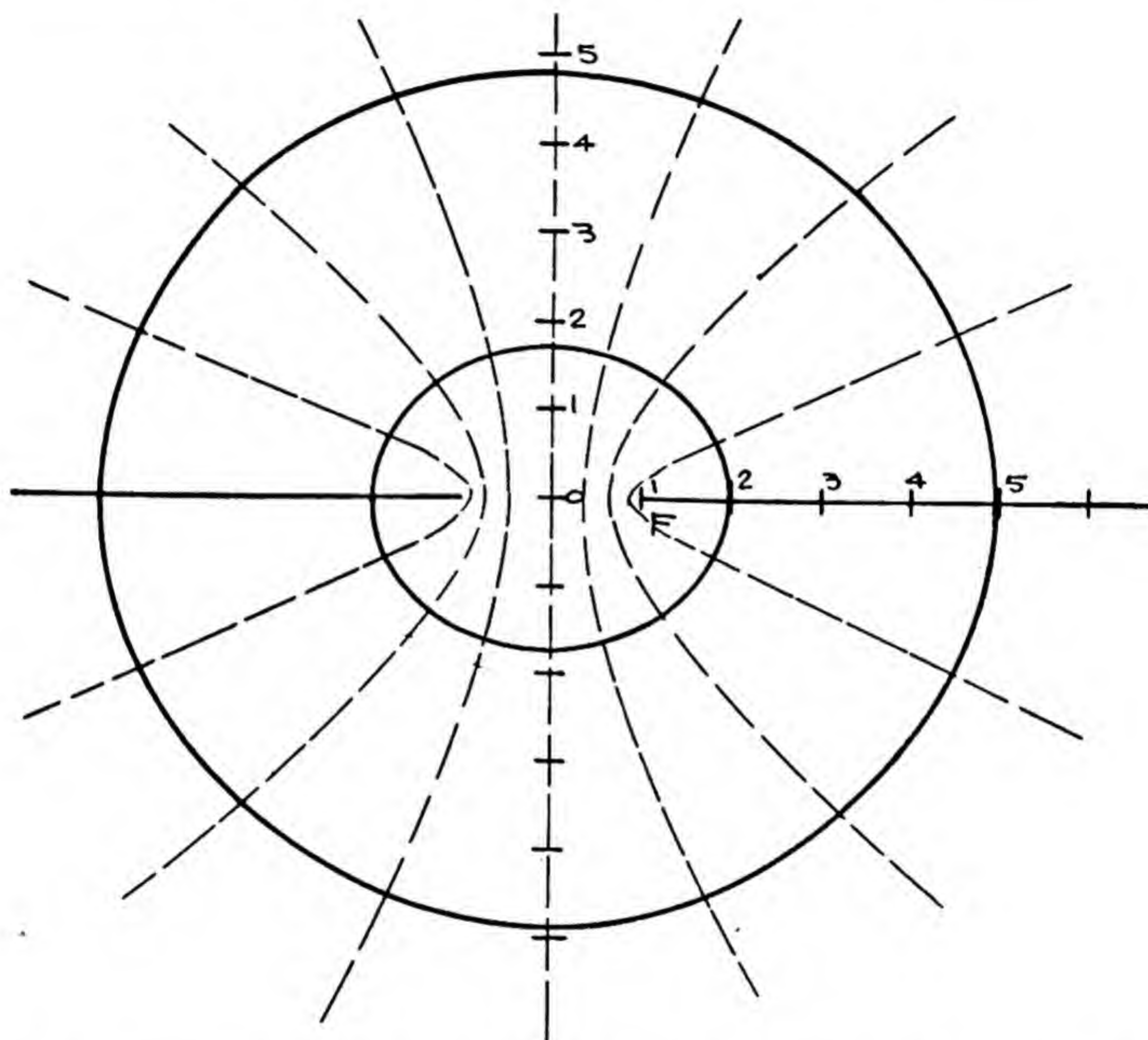


FIG. 4.1—Diagram of the ellipse family which satisfies Stefan's conditions for diffusion proportional to diameter.

The proportionality of small-pore diffusion rates with pore diameter can be demonstrated with simple equipment. The data in Figure 4.2 were obtained with pores drilled in copper discs 0.8 mm. thick and sealed to sample bottles containing water or alcohol to a level of 1 cm. below the rim. The bottles were weighed periodically to determine rate of vapor escape through the pores. The graph shows that the spherical shape of diffusion shells was maintained in a quiet room over pores having a diameter as large as 1.27 cm. The diffusion gradient of alcohol was more than four times that of water,

but the diffusion curves obtained showed the same type of proportionality. This indicated that the vapor pressure of the diffusing substance was not a factor in determining the shape of the diffusion shells.

When Browne and Escombe (1) applied the diameter law to leaf stomata, however, another discrepancy arose. If stomata act independently and have a diffusive capacity proportional to their diameter, then the diffusive capacity of a leaf would be many

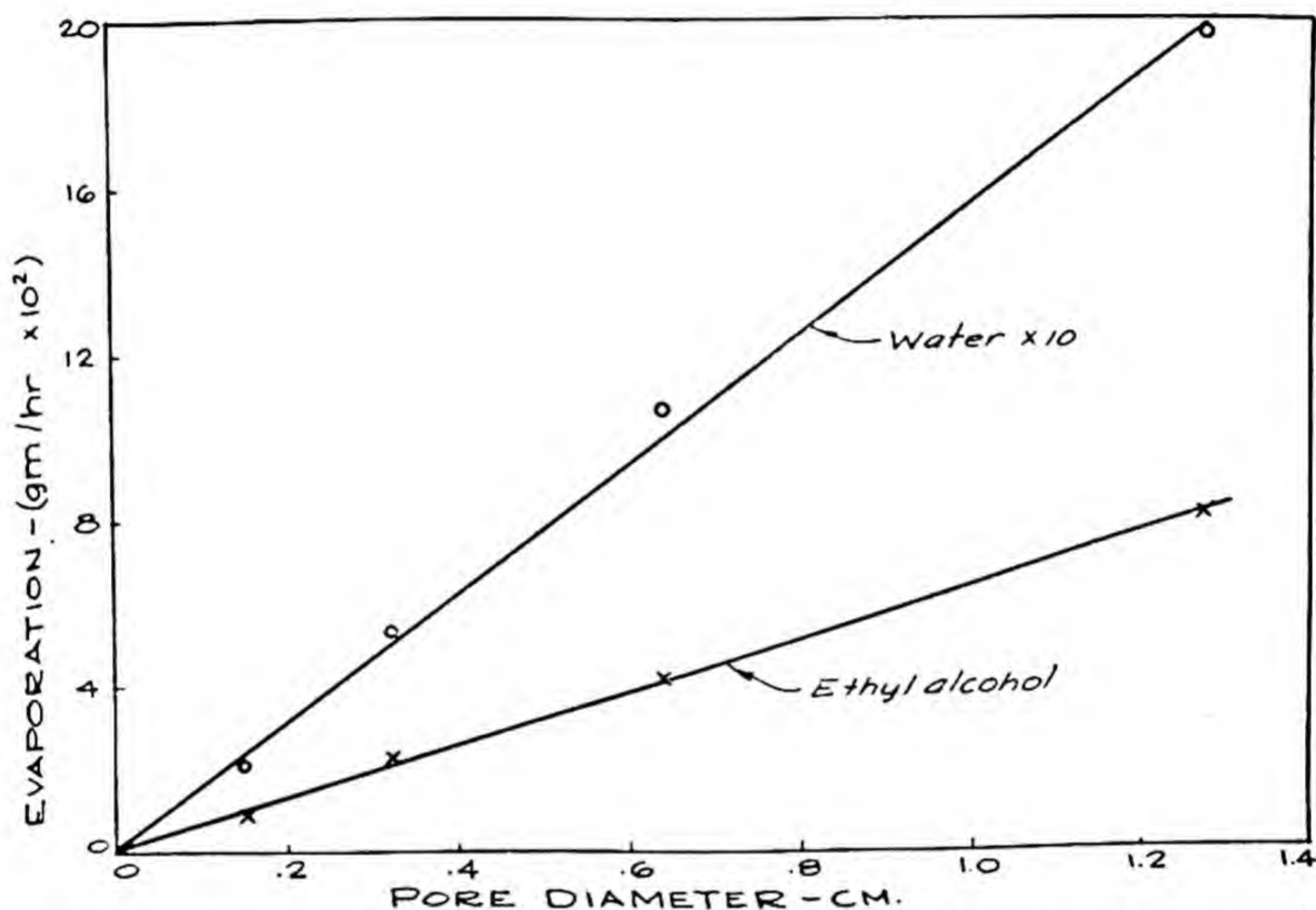


FIG. 4.2—Relation of diffusion through small pores to pore diameter.

times greater than the maximum capacity of a free diffusing surface having the same size as the leaf. This discrepancy may be explained by assuming that stomata do not act independently but interfere with each other; that numerous small pores in a septum cause a mutual gradient reduction which prevents their attaining the diffusion rates possible for isolated pores.

Although the phenomenon of interference was recognized as early as 1900, no experiments designed to ascertain the laws governing its behavior were reported prior to 1930. Browne and Escombe reported that interference was negligible when pores were spaced more than ten diameters apart. Their data were obtained with pores of 0.38 mm. diameter. They assumed that this relationship was independent of pore size, hence their assumption that stomata act

independently. Their experimental pores were more than 1,000 times the area of a typical plant stoma.

Jeffreys in his treatment of evaporation (4) seriously questioned the validity of Browne and Escombe's assumption. He emphasized the importance of interference by showing that the diffusive capacity of a leaf, as calculated on the assumption that stomata act independently, would maintain a vapor concentration outside the leaf equal to 300 times that of atmospheric saturation. By a different method of calculation he showed that the open stomata of a leaf acting independently could maintain atmospheric saturation over the outer surface of the leaf if there were only 600 stomata per square centimeter. The plant species he used as an example has about 30,000 stomata per square centimeter of leaf surface. Jeffreys' studies, although they drew attention to the apparent importance of interference, did not include attempts to define interference laws or experiments to measure the effect of interference.

The work of Huber (3) published in 1930, included the first set of experiments designed to determine the laws governing interference. His results are presented graphically in Figure 4.3. He graphed the evaporation per septum, expressed as a fraction of the free-surface evaporation rate, against the total pore area, expressed as a fraction of the pore-bearing surface. The graph shows that the total septum evaporation approached free-surface evaporation with increasing rapidity as pore size decreased. Huber also emphasized that, although small-pored-septum evaporation approached the free-surface evaporation rate when only a small fraction of the total septum area was occupied by pores, yet it never reached or exceeded this rate. In his attempt to formulate a mathematical expression for interference, Huber assumed that the curves in his graph were segments of symmetrical hyperbolas, and obtained an empirical constant, the hyperbolic constant, which would predict the entire evaporation curve if a single point on the curve were known. He expressed his interference equation as:

$$\left[\frac{1}{1-x} - 1 \right] \cdot \left[\frac{y}{1} - 1 \right] = C \quad (2)$$

in which x and y are the abscissa and ordinate values in the graph, and C is the hyperbolic constant. In applying this equation to his data he found that predicted values did not differ from observed values by more than the error present in his experimental determinations. His equation, however, is an empirical one, and it sheds no light on the physical interpretation of interference. Moreover, it depends on the validity of the assumption that the curves are

segments of symmetrical hyperbolas. An examination of Figure 4.3 shows that only the curve for the largest pores has data points on the upper half of the curve, and this curve segment is so fragmentary that it can hardly be relied upon to establish Huber's assumption.

DERIVATION OF AN INTERFERENCE LAW

A theoretical interference law which fits experimental data may be deduced from a consideration of the diffusion shells associated

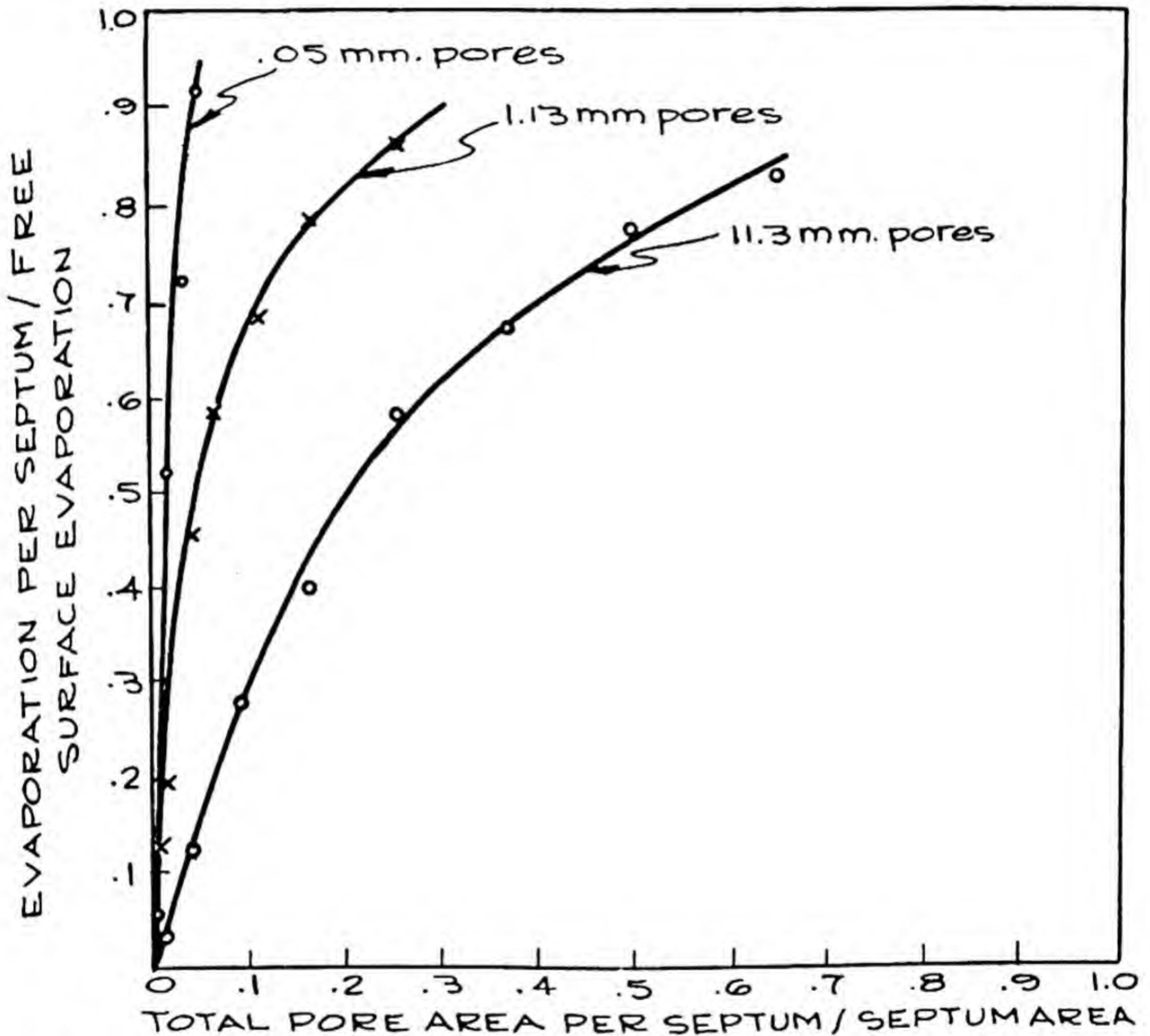


FIG. 4.3—Huber's data for diffusion through multiperforate septa.

with Stefan's analysis of single pore diffusion and from the disturbances these shells suffer when many pores initially far apart are brought close together. When the pores in a septum are far apart they act independently, and the total septum rate of diffusion is the sum of the diffusion rates of the individual pores. Furthermore, at distances from a given pore of several pore diameters, the diffusion shells around the pore will be spheres (Fig. 4.1). Consequently, the

vapor concentration in the vicinity of a pore varies inversely as the square of the distance from the pore. This is a relationship analogous to the dependence of light intensity on the distance from a point source of light.

When the spacing between pores becomes small, there results (a) an increase in the vapor concentration above a given pore because of the vapor flow from neighboring pores and (b) a decrease in the vapor concentration below the pore because some of the vapor originally available to the given pore has gone out through neighboring pores. Both effects are directly dependent on the rates of diffusion of the neighboring pores.

From the above discussions it may be concluded that when the distance between pores is changed, the change in the rate of diffusion of a given pore, dQ , is (a) dependent directly on the diffusion rate Q of the neighboring (interfering) pores and (b) dependent directly on the change in the inverse square distance between pores $d\left(\frac{1}{D^2}\right)$. We have then

$$dQ = -k Q d\left(\frac{1}{D^2}\right) \quad (3)$$

where D is the distance between pores, k is a constant of proportionality, and the negative sign means that an increase in $\frac{1}{D^2}$ causes a decrease in Q . The expression (3) may be rewritten as

$$\frac{dQ}{d\left(\frac{1}{D^2}\right)} = -k Q \quad (4)$$

which is analogous to the equation describing the rate of growth of bacteria. In order to integrate equation (3) it should be written in the form

$$\frac{dQ}{Q} = -k d\left(\frac{1}{D^2}\right) \quad (5)$$

which integrates directly to

$$\log Q = -\frac{k}{D^2} + C. \quad (6)$$

The constant of integration, C , is to be identified with the log of the isolated pore rate by simply specifying that $Q = Q_1$, the rate for an isolated pore, when D is very large. Hence,

$$\log Q = \log Q_1 - \frac{k}{D^2}, \text{ or} \quad (7)$$

$$\log \frac{Q}{Q_1} = \frac{-k}{D^2}. \quad (8)$$

If equation (8) is written in the exponential form, it becomes

$$Q = Q_1 e^{\frac{-k}{D^2}} \quad (9)$$

Expansion of the exponential term into an infinite series gives

$$Q = Q_1 \left[1 - \frac{k}{D^2} + \frac{1}{2} \frac{k^2}{D^4} - \frac{1}{6} \frac{k^3}{D^6} + \dots \right] \text{ or} \quad (10)$$

$$Q = Q_1 - Q_1 \left[\frac{k}{D^2} - \frac{1}{2} \frac{k^2}{D^4} + \frac{1}{6} \frac{k^3}{D^6} - \dots \right]. \quad (11)$$

From (11) we see the diffusion through a pore in a multiperforate septum is equal to the diffusion through an isolated pore less the interference,

$$Q = Q_1 - I. \quad (12)$$

However, I is not conveniently calculated because it involves interference between a given pore and the second, third, and succeeding rows of surrounding pores, as well as the first row. The importance of this concept of infinite series interference can be emphasized by reference to Figure 4.2. Spherical diffusion shells were established over pores 1.27 cm. in diameter in an open laboratory. The size of these shells is thus to be measured in centimeters, a distance which might easily involve twenty or more concentric rings of interfering stomata on a leaf.

EXPERIMENTAL CONFIRMATION OF THE INTERFERENCE LAW

In applying the interference law to experimental measurements of multiperforate septum diffusion, data of Weishaupt (11) and of the author (10) are used. Weishaupt's septa contained pores 0.3 mm. in diameter laid in a hexagonal pattern, and the pore-bearing area was kept constant as pores were brought closer together. The author used two series of septa, one with pores of 0.2 mm. diameter and the other of 0.3 mm. diameter. These pores were laid in a hexagonal pattern and pore number was kept constant as pores were brought closer together. Rates of liquid evaporation through the septa were measured by weighing, periodically, the liquid-filled containers to which septa had been sealed. Figures 4.4 and 4.5 are graphs of diffusion rate per pore against distance between pores. Figure 4.5 includes three of several such curves obtained by Weishaupt. The rate per pore decreased as pores were brought closer

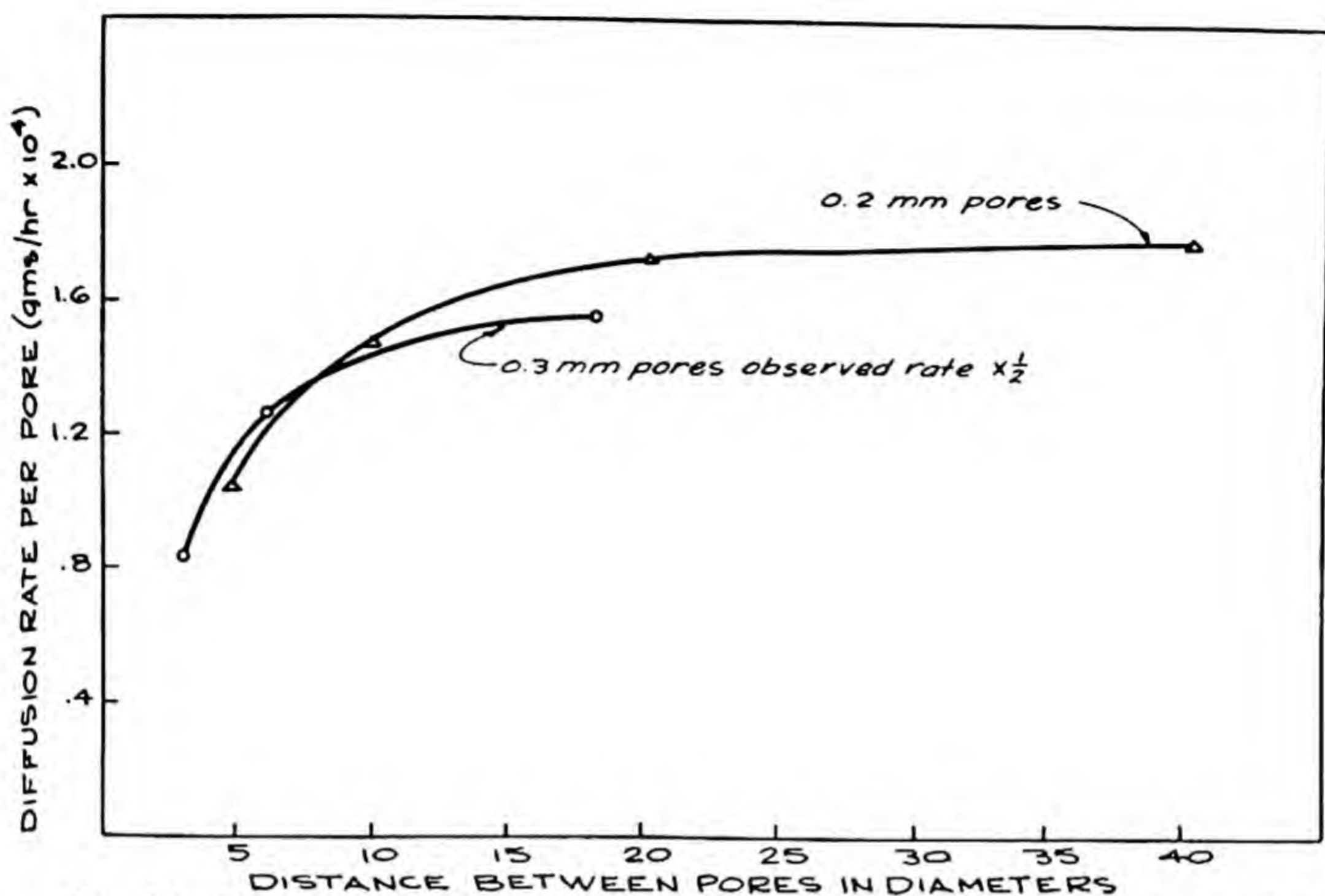


FIG. 4.4—Interference in multiperforate septa with diameters of 0.2 and 0.3 mm.

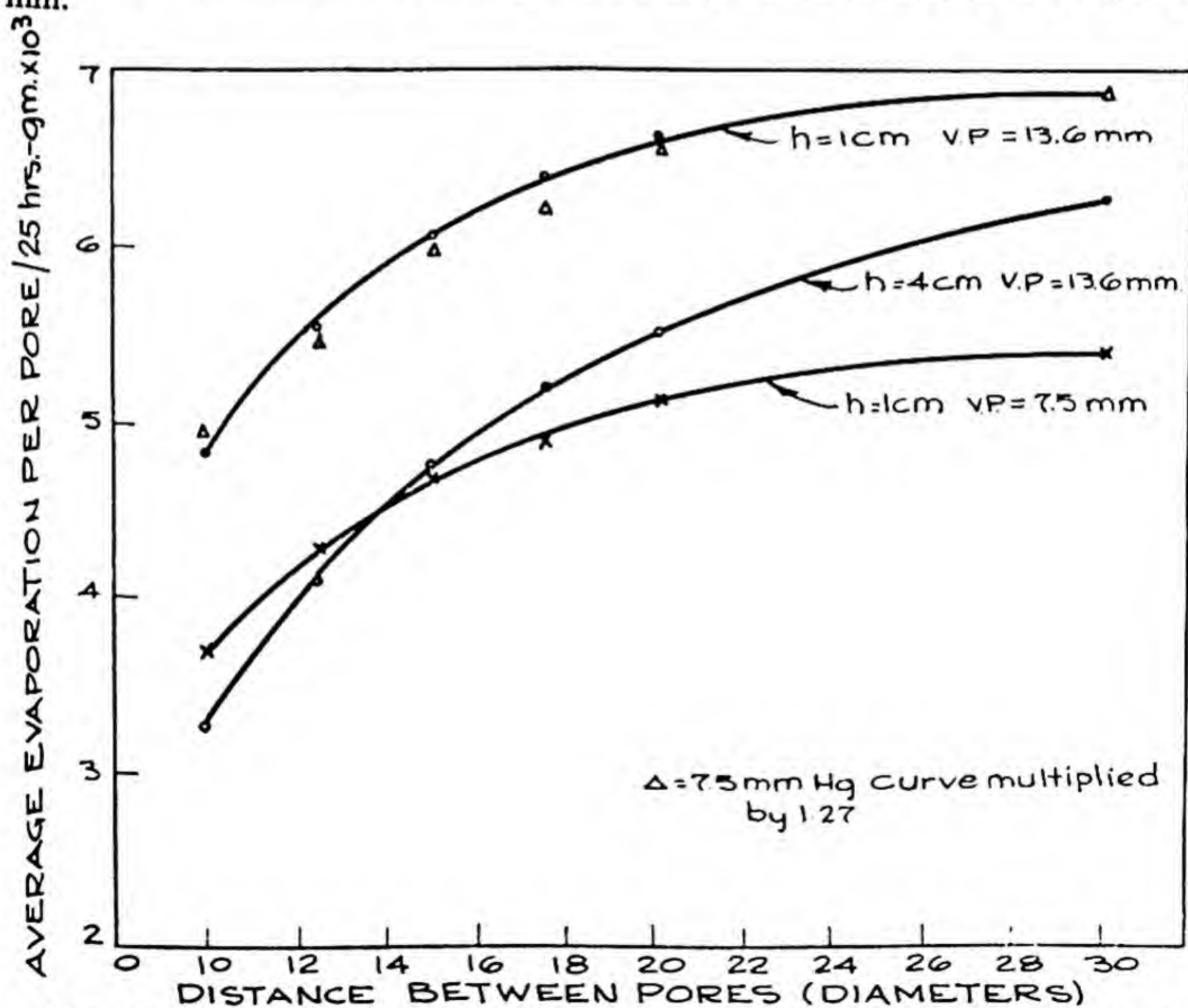


FIG. 4.5—Interference in multiperforate septa with pore diameters of 0.3 mm. Data of Weishaupt. H = height of membrane above liquid; VP = vapor pressure deficit of atmosphere.

together. Even these relatively large pores showed marked interference beyond the ten-diameter spacing where Browne and Escombe (1) regarded interference as negligible.

Figures 4.6 and 4.7 are graphs of the logarithms of observed diffusion rates against the inverse square of distance between pores $\left[\log Q \text{ against } \frac{1}{D^2} \right]$. This form follows from equation (7) because

Q_1 is constant for any given series of septa. Four of the curves obtained by Weishaupt under various conditions are graphed in

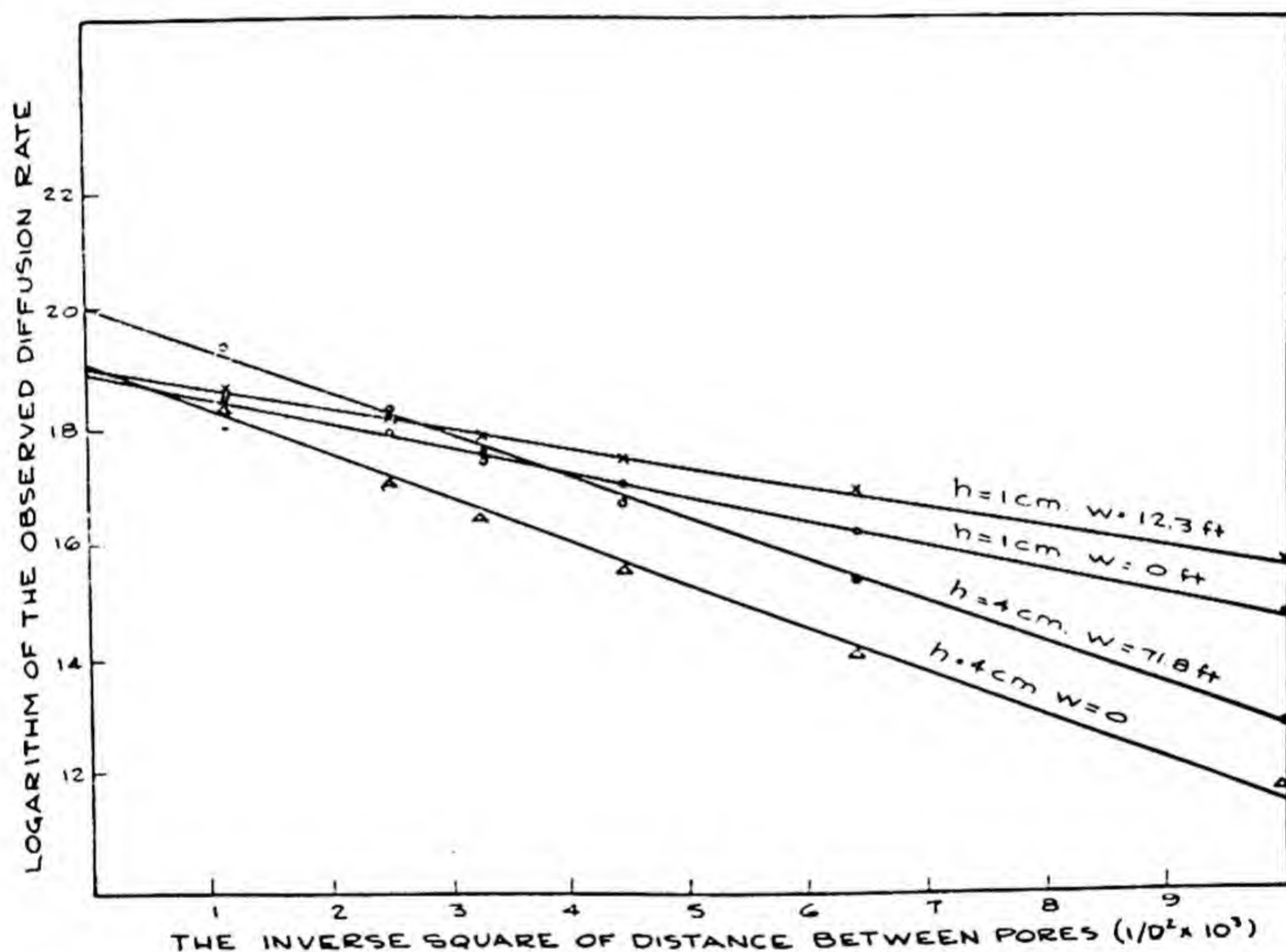


FIG. 4.6—Logarithm of diffusion per pore plotted against the inverse square of the distance between pores. Pore diameters 0.3 mm. Data of Weishaupt. H = height of membrane above liquid; W = wind velocity in ft./min.

Figure 4.6. A reasonably good fit is obtained under every condition of the experiments, indicating that changing the experimental conditions merely changed the value of k in equation (7). Our own data (Fig. 4.7) agree, with the exception of one data point (not shown) where 37 pores were spaced 3 diameters (0.9 mm.) apart so that the entire pore-bearing area was less than 1 cm. in diameter. Under these conditions the group of pores tended to act as a single pore and interference was reduced below the expected value.

THE EFFECT OF PORE SIZE ON INTERFERENCE

We have shown that diffusion through a series of multiperforate membranes of uniform pore size but varying spacing can be described by the equation

$$\log Q = \log Q_1 - \frac{k}{D^2}, \quad (7)$$

and that 0.2 or 0.3 mm. pores show a very considerable interference at a spacing of 10 diameters, a distance ratio at which Browne and Escombe (1) assumed no interference. Before we can predict inter-

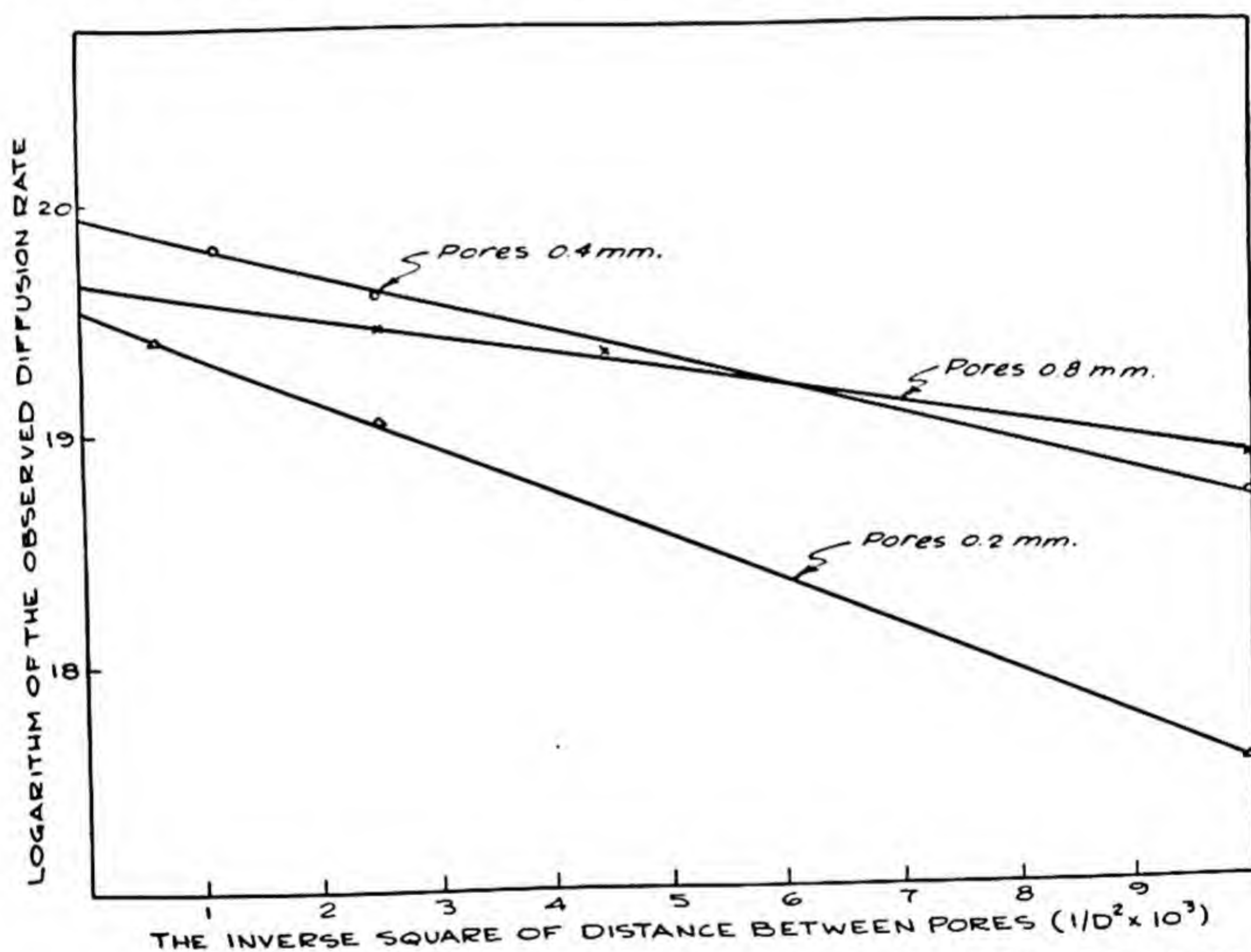


FIG. 4.7—Logarithm of diffusion per pore plotted against $1/D^2$ (cf. Table 4.1).

ference between stomata on a leaf, however, where pores are $10\text{--}20\mu$ instead of 200μ , when fully open (Table 4.2) we must know something of the effect of pore size and absolute distance between pores on interference.

The data of Table 4.1 show results with three series of membranes in which the pore diameter (a) varied from 0.2 to 0.8 mm. and pore distance (D) from 2 to 16 mm. Values for Q , diffusion per pore, were obtained experimentally, and show diameter proportionality. The k values vary, of course, between membranes, but show

satisfactory uniformity, with the single exception of the value for the 0.2 mm., 40-diameter membrane. The values for Q/Q_1 indicate that interference was *not* independent of pore size over this range for either the 10- or 20-diameter spacings, but tended to increase as pore size and the absolute distance between pores decreased. Various extrapolations can be made from the data of Table 4.1, but none of

TABLE 4.1
EFFECT OF PORE SIZE ON INTERFERENCE

Pore Spacing in Diam.	Pore Diameter (a)								
	0.8 mm.			0.4 mm.			0.2 mm.		
	Q	k	Q/Q_1	Q	k	Q/Q_1	Q	k	Q/Q_1
10.....	6.60	4.93	.925	3.24	1.98	.884	1.44	.824	.814
15.....	6.90	4.61	.968
20.....	7.00	4.61	.981	3.55	2.11	.967	1.68	.816	.949
30.....	3.62	2.02	.986
40.....	1.74	.960	.983
$\infty = Q_1$	7.13	3.67	1.77
Ave. k	4.72	2.04863
k/a	5.90	5.10	4.31

them give reliable estimates of diffusion with pores of stomatal size. The value of k/a appears to be most promising, but its downward trend, which is obviously nonlinear, has not been sampled over a wide enough range to warrant predictions of its value at stomatal sizes.

We may assume that the diffusion of CO_2 into a leaf or of water from a leaf cannot exceed the exchange with a free absorbing or evaporating surface at the same diffusion gradients (3). Since extrapolation of diameter law diffusion to stomatal sizes and numbers gives values ten to fifty times this limit, it seems clear that either interference reaches a high value with small pores, even though the relative spacing is still 10 diameters, or diffusion through pores does not follow the diameter law at these pore sizes. The available data do not permit a choice between these alternatives. Although the first seems more probable, the two factors may act together.

IMPLICATIONS OF MULTIPERFORATE SEPTUM DIFFUSION FOR
PLANT PHYSIOLOGY

Attempts to correlate the rates of photosynthesis or transpiration with stomatal number or aperture have met with indifferent success. A survey of stomatal characteristics among land plants shows that plants living under very similar environmental conditions have widely varied stomatal sizes and densities without showing any apparent advantages or disadvantages relatable to these characteristics. In Table 4.2 a list of stomatal characteristics for a variety of plants is presented, showing density and size of fully open stomata as determined by Eckerson (2). The other characteristics shown were calculated from these data. Inspection of this table shows that such stomatal characteristics as density, linear dimensions, average spacing distance, perimeter, and effective diameter show wide variation. However, the relative distances between stomata (that is, the distance expressed in effective diameters of the stomata), show much less variation. This suggests that relative spacing may be more important in determining diffusive capacity than the other characteristics. The largest effective diameters in this table are about one-half the size of Huber's 0.05 mm. pores, and the widest relative spacing is about twice that in which he obtained diffusion rates equalling 92 per cent of free surface diffusion. The relative stomatal spacing for all the leaves in this table would seem to favor diffusion rates closely approximating those of a free surface. It is not surprising, therefore, that wide variations in stomatal sizes and densities show low correlation with observed diffusion rates.

Studies of diffusion under varied degrees of stomatal opening have shown a marked lack of correlation between observed diffusion rates and apparent stomatal aperture. These observations have raised doubts concerning the effectiveness of stomata as a diffusion regulating mechanism. Some observations of natural phenomena serve to heighten these doubts; for example, many plants show apparent stomatal closure during mid-day with but slight diminution of gas exchange. The stomata of maize seldom appear to be open yet this plant ranks high in rates of CO_2 absorption.

Sayre (6) compared transpiration with evaporation and showed that with open stomata transpiration equalled 89.6 per cent of black surface evaporation, while night closure of stomata only reduced transpiration to 33.7 per cent of black surface evaporation. Sayre explained the high diffusive capacity of apparently closed stomata by correlating diffusion with the perimeter of an elliptical pore. He pointed out that closing a stoma diminishes its perimeter only slightly, although its area is much smaller than that of an open

TABLE 4.2
STOMATAL CHARACTERISTICS OF THIRTEEN PLANTS
CALCULATED FROM DATA OF ECKERSON

Plant	Density Stomata per sq. cm. of leaf surface	Size Length (2a) x breadth (2b) in microns	Spacing Average distance between stomatal centers in microns	Perimeter Perimeter of an open stoma $2\pi\left[\frac{a^2 + b^2}{2}\right]^{1/2}$ in microns	Effective Diameter Diameter of a circle of equal perimeter in microns	Relative Spacing Distance between stomata in effec- tive diameters
Bean <i>Phaseolus vulgaris</i>	28,100	7x3	67.5	16.8	5.36	12.6
Begonia <i>Begonia coccinea</i>	4,000	21x8	179.0	49.0	15.58	11.5
Castor bean <i>Ricinus communis</i>	17,600	10x4	85.0	23.9	7.60	11.2
Coleus <i>Coleus blumei</i>	14,100	10x5	95.2	24.8	7.90	12.0
English ivy <i>Hedera helix</i>	15,800	11x4	90.0	26.0	8.27	10.9
Geranium <i>Pelargonium domesticum</i>	5,900	19x12	146.0	50.0	15.90	9.2
Maize <i>Zea mays</i>	6,800	19x5	137.0	43.6	13.89	9.9
Nasturtium <i>Tropaeolum majus</i>	13,000	12x6	99.1	28.0	8.91	11.1
Oat <i>Avena sativa</i>	2,300	38x8	235.8	86.5	27.50	8.6
Sunflower <i>Helianthus annuus</i>	15,600	22x8	90.5	52.0	16.50	5.5
Tomato <i>Lycopersicon esculentum</i>	13,000	13x6	99.2	32.8	10.42	9.5
Wandering Jew <i>Zebrina pendula</i>	1,400	31x12	302.0	74.0	23.55	12.8
Wheat <i>Triticum sativum</i>	1,400	38x7	302.0	86.0	27.35	11.0
Average	10,692	19x7	148.3	45.6	14.52	10.4

stoma. He accepted Browne and Escombe's assumption that stomata act independently.

In the light of the evidence brought together in the present report it is clear that interference cannot be neglected in discussions of leaf diffusion. Multiperforate septa having pores 0.2 mm. in diameter show diffusion rates at the 10-diameter spacing, equaling about 81 per cent of the isolated pore rate. Since interference appears to be relatively greater for smaller pores, the mutual interference exercised by plant stomata (which have linear dimensions of about one-twentieth the diameter of these artificial pores) must cause reductions that bring diffusion down to a fraction of the isolated pore rate. Such interference would largely account for the lack of correlation between observed diffusion rates and degrees of stomatal aperture.

Figure 4.8 shows how this latter effect is brought about. This graph is patterned after the curves obtained with 0.3 mm.- and 0.2 mm.-pored septa. The lower curve is drawn to represent pores one-half the diameter of those in the upper curve. Consider stomata at a spacing of five diameters, fully open, which have a diffusion rate represented by point *A* on the upper curve. If such stomata close until their effective diameter is one-half the value when fully open, then the relative spacing changes from five to ten diameters and their diffusion rate is found at point *B* on the lower curve. The rate at point *B* is 80 per cent of the rate at *A*, instead of 50 per cent, as independent action of stomata would predict. The fading of interference during stomatal closure would tend to hold diffusion rates, and thus photosynthesis or transpiration, at a higher level than is indicated by the effective diameter of stomata.

In this discussion nothing has been said about another stomatal characteristic—the thickness of the guard cells as compared to the breadth of the stomatal aperture. That is, a stoma can be regarded as a tube, and the ratio of tube length to tube width at different degrees of opening may have as critical an influence as any of the other characteristics here discussed. The work of Renner (5) with models of sunken stomata showed that such lengthening of the diffusion path might reduce the gradient from 30 to 70 per cent, but the effect of the ratio of tube length to stomatal breadth needs to be studied more exhaustively, with special regard to its influence in the range of stomatal dimensions.

SUMMARY

1. Diffusion through small pores is proportional to pore diameter rather than area, and Stefan's analysis indicates that the spheroidal shape of diffusion shells is responsible for this proportionality.

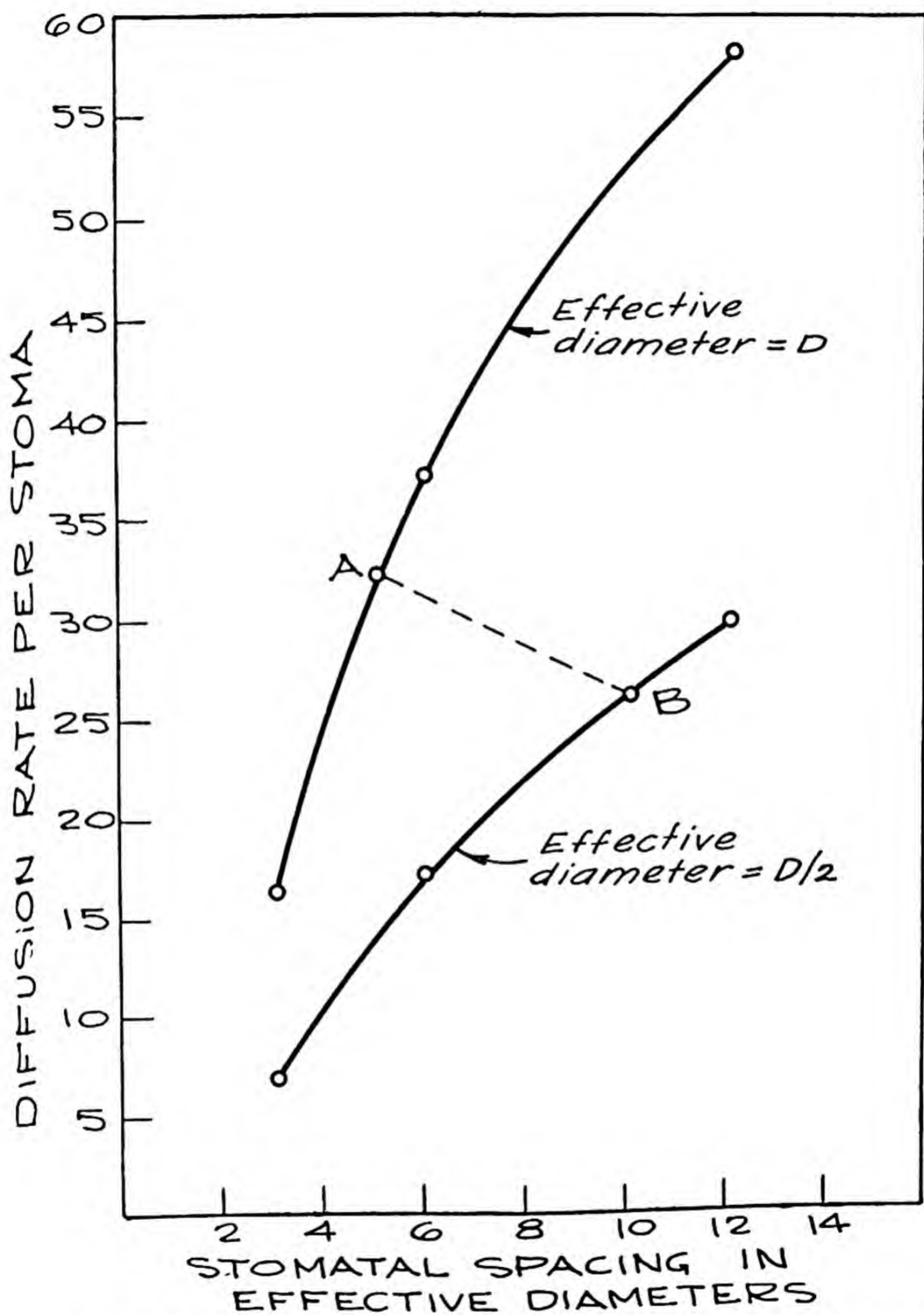


FIG. 4.8—Effect of reduced interference on diffusion rate during stomatal closure.

2. The concept of spheroidal diffusion shells suggests that interference between neighboring pores in a septum should be inversely proportional to the square of the distance between pores (D^2) and directly proportional to the diffusion rate of neighboring pores (Q). A theoretical interference equation is derived which shows that the relation between diffusion rate per pore (Q) and distance between pores (D) is given by

$$\log Q = \log Q_1 - \frac{k}{D^2} \quad (7)$$

where Q_1 is the diffusive capacity of an isolated pore and k is a proportionality constant.

3. Several sets of experimental results provide confirmation of the interference equation. In graphs of $\log Q$ against $\frac{1}{D^2}$ straight lines were obtained.

4. A comparison of interference exhibited by septum series of varying pore diameters showed that interference became relatively greater as pore size decreased, suggesting that interference may cause important reductions in stomatal diffusive capacities. The fading of interference during stomatal closure would tend to maintain a higher diffusive capacity than relative stomatal aperture would predict.

5. A comparison of stomatal characteristics of thirteen plants shows that relative distance between stomata (distance expressed in effective stomatal diameters) shows the least variation, and may be more important in determining the diffusive capacity of leaves during photosynthesis and transpiration than other characteristics.

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5.

The Chloroplasts: Their Structure, Composition, and Development

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CHLOROPLASTS are located and photosynthesis in vascular plants carried on typically within the leaf. We may, therefore, consider briefly the structure of the generalized mesophytic leaf as it relates to photosynthetic function. The leaf is an organ whose structure is specialized so that it provides the maximum area in proportion to mass for the absorption of sunlight. However, in respect to its surfaces, the leaf structure is a compromise between two opposing tendencies; namely, the tendency to absorb CO_2 by exposing the photosynthesizing cells to the atmosphere, and the tendency to protect the leaf cells from being desiccated by the atmosphere.

The generalized leaf consists of several somewhat compact layers of mesophyll cells (the palisade parenchyma) toward the upper surface and several layers of loosely arranged mesophyll cells (spongy parenchyma) below them. All the parenchyma cells are arranged sufficiently loose so as to be in contact at some cellular surface with the air spaces of the leaf. The air spaces serve for the rapid exchange of gases, diffusion being very rapid in the gaseous state as compared with diffusion in the liquid state (1). The spongy mesophyll cells, in contrast to the palisade cells, are in fairly close association with vascular bundles or bundle ends through which substances are transported in the dissolved state to and from the cells. The development of intercellular spaces and the compactness of cellular arrangement are probably related, in part, to the auxin content of the developing mesophyll cells and the effect of light in destroying the auxin. Thus, leaves developing in strong sunlight may have more compact mesophyll tissue with fewer air spaces, and leaves developing in the shade may have palisade cells, which appear more like spongy mesophyll.

The mesophyll cells contain numerous chloroplasts which are green disk-like structures of about 5μ in diameter. They lie embedded in cytoplasm and appressed with their broad sides parallel to the cell walls. In *Ricinus communis* Haberlandt (2) estimated that there were a total of 403,000 chloroplasts per square millimeter of leaf area, some 92,000 or 18 per cent of these being in the spongy parenchyma; on the average, a palisade cell contains about thirty-six chloroplasts and a spongy mesophyll about twenty.

In a number of plants there appears to be a well developed tendency for the chloroplasts to become arranged in the cells so as to receive maximum light at lower light intensities; that is, by arranging themselves about the edge of the cell facing the light with their flat sides facing against this wall. At high light intensities the plastids may move so that the edges rather than the broad faces of the plastids receive the light (3, 4).

The thin leaf is covered by a layer of epidermal cells both on the upper and lower surfaces. These cells have a waxy coating, the cuticle, which reduces evaporation of water. It is estimated that not more than 10 per cent of the water evaporating from the leaf escapes by way of the cuticle. The epidermal cells contain small plastids, in general poorly developed and devoid of chlorophyll. Besides these cells the epidermal layers contain specialized pairs of epidermal cells, the guard cells, which surround openings that lead into the mesophyll spaces. The openings, or stomata, occupy typically less than 1 per cent of the surface area of the epidermis.

The guard cells represent a mechanism for regulating the size of the stomatic orifice through which direct gaseous exchange occurs between the intercellular mesophyll spaces and the outer air. The guard cells are generally characterized by special thickenings of their cell walls and special relations with their neighboring epidermal cells (2). These anatomical features cause the stomata to open when the guard cells become turgid and to close when they lose their turgidity. The guard cells, in contrast to the other epidermal cells, have functional green plastids which generally contain starch (even at a time when the parenchyma cells may lack starch). The osmotic value of the other epidermal cells is relatively constant and always lower than that of the guard cells. The osmotic value of the guard cells, however, is not constant. It is lowest when the stomata are closed and highest (some 2-10 atmospheres more than in the epidermal cells) when the stomata are opened.

When the guard cells of a leaf are exposed to light in the morning, their pH increases (5), their starch content decreases, the osmotic value of their cell sap increases, and the stomata open. At night the stomata of the majority of plants become closed, but not necessarily so completely as entirely to prevent gaseous exchange

with the outside air. In the presence of factors which bring about rapid evaporation (i.e., strong sunlight, low humidity) the stomata become effective in regulating water loss only when they are almost closed. If the supply of water reaches a certain minimum which causes the guard cells to lose turgidity in spite of their higher osmotic pressure, they may close regardless of other factors. A hypothesis which has been suggested to explain these facts is that the control of the opening and closing of the stomata is a result of the osmotic changes in the guard cells, the osmotic changes being primarily influenced by the sugar-starch equilibrium, or more directly by factors affecting the glucose-1- PO_4 -phosphorylase-starch system.

MORPHOLOGY OF THE CHLOROPLASTS

GENERAL MORPHOLOGY

The chloroplasts are well-defined cytoplasmic bodies containing chlorophyll and carotenoid pigments. These bodies appear to be able to carry on some of the first stages in photosynthesis apart from the other cell constituents. The chloroplasts are present in all cells that carry on photosynthesis, with the exception of the photosynthetic bacteria and the blue-green algae. In the blue-green algae chlorophyll is diffusely distributed throughout the peripheral cytoplasm of the cell, perhaps in the form of grana as suggested by Geitler (6).

In some species of the higher algae there may be one or two chloroplasts in a cell; in other species there may be some ten or more chloroplasts per cell. The chloroplasts are usually placed parietally just beneath the cell wall, embedded in cytoplasm, as in the higher plants; they may be cup-shaped as in *Chlorella*, or spiral-shaped as in *Spirogyra*, or like a network, as in *Oedogonium*; or they may be suspended in the center of the cell as in the stellate-shaped chloroplasts of *Zygnema*. The cup-shaped chloroplast of *Chlorella* may often appear smooth, but if grown in the dark in the presence of glucose it may appear to be fragmented.

In many of the algae pyrenoids are present in the chloroplasts. The pyrenoids are spherical bodies, said to be rich in protein, embedded in the matrix of the chloroplasts. Their function is unknown. They may represent a concentration of enzymes possibly related to carbohydrate synthesis, since, in the green algae at least, starch grains develop in their immediate vicinity, forming a kind of sheath around the pyrenoid. Pyrenoids may be present or absent in closely related forms. They have been reported to originate *de novo* or by division; they may divide without the occurrence of cell division, and their number may be conditioned in some cases by the nutritional state of the cell. Starvation frequently causes

pyrenoids to disappear or they may increase in number if cells are well nourished. There may be one pyrenoid per chloroplast as in *Chlorella* or *Zygnema*, or a larger number as in *Spirogyra*.

Some algae contain eyespots. An eyespot may appear as a reddish or brownish-red dot or streak in the anterior region of the cell adjacent to the surface membrane and protruding from the surface of the chloroplast. If several chloroplasts are present per cell, then only one of them may contain the eyespot. It is probable that the eyespot is formed by the chloroplast, since the pigments of the eyespots are carotenoids, and carotenoids are known to be produced in plastids. The eyespot of *Euglena*, however, is formed independently of the chloroplasts (7).

In the higher plants the chloroplasts are generally uniform in size. In about seventy-five of the higher plants that have been examined they average some 4–6 μ in diameter. In the palisade cells they often are packed so closely as to make an almost compact layer on the inside of the cell wall. In general, the chloroplasts of "shade" plants are larger and richer in chlorophyll, and they contain a higher proportion of chlorophyll *b* to chlorophyll *a* than do the "sun" plants (8).

The cells of many plants appear to have the ability to regulate the number of plastids per cell; i.e., there is a relative cell constancy. If the chloroplasts are few, division of the chloroplasts may take place; if many are present, a degeneration ensues. In certain strains of maize, Eyster (9) noted that if cells have large plastids, there are fewer plastids per cell than if cells contain smaller plastids.

There is evidence that in the enlarging leaf cells chloroplasts may divide by a process of constriction. Heitz (3), in 1922, observed that in the enlarging cells of intact leaves of *Funaria* and *Mnium*, plastids were still multiplying, the total time required for division of a chloroplast being about eight days. The division generally occurs by elongation of a plastid and pinching off in the center. In a few cases the region of elongation may appear as a colorless zone and finally pinch off (3).

DETAILS OF STRUCTURE

The literature on the structure of the chloroplasts as revealed by the light microscope has been amply reviewed in recent years by Rabinowitch (8), Weier (10), and Frey-Wyssling (11). In intact cells, such as the leaf parenchyma of tomato, spinach, etc., the chloroplasts appear to be saucer-shaped with their concavity facing the cell vacuole. They may appear completely homogeneous or may contain very fine granules. On injury of the cell, the chloroplasts may take on a more or less coarsely granular appearance, or at

times they may take on a foamy appearance in which no distinct grana are observable.

By tearing the cells apart in a hypertonic sucrose solution, the chloroplasts float out into the solution, and may be seen to retain their smooth appearance for several minutes or longer, gradually taking on a granular appearance. If the chloroplasts are floated out into distilled water or isotonic saline, they take on a distinctly granular appearance in several minutes; at the same time, vesicles or blebs are observed to form as shown in Figure 5.5. One or several large blebs which develop seem to have their origin on the concave side of the chloroplast where a starch vacuole appears to be localized. Smaller, numerous blebs which form over the surface of the chloroplast may represent the swelling of units originally surrounded by membranes, or may be precipitation membranes formed on injury. From the fact that chloroplasts, maintaining a coherent structure, can be removed from the cells without adhering cytoplasmic films, and that the chloroplasts show osmotic behavior, a limiting osmotic membrane surrounding the chloroplasts is postulated.

In some plants the vacuoles in which starch forms appear to be more readily observable as tiny colorless areas. Recently Rhoades and Carvalho (12) investigated chloroplasts in corn. The dark green chloroplasts in mesophyll cells of corn normally do not form starch unless supplied with a high sucrose concentration. However, the single layer of cells surrounding the vascular bundle of corn, the parenchyma sheath, contains chloroplasts especially adapted to the rapid temporary storage of starch. These cells contain pale green chloroplasts, each with an average of twenty to forty ellipsoidal starch grains. The pale green outer area of the chloroplast contains the embedded starch granules; an even paler green central area contains neither starch grains nor vacuoles. When the rate of movement of sugars into these sheath cells is slower than the rate with which the sugars move out into the vascular bundles, the starch disappears. The pale green chloroplasts of the parenchyma sheath cells, free from starch, are then seen to contain approximately the same number of vacuoles as originally of starch grains. Zirkle (13) also noted in the mesophyll plastid of *Elodea canadensis* a centrally placed vacuole normally containing a single starch grain. This vacuole remained visible when no starch was present. How constant these starch vacuoles are in chloroplasts of other plants is a problem requiring further study.

The granules appearing in the chloroplasts were first observed in 1883, by Meyer, who called them "grana." They were later rediscovered by Dautreline (14) and Heitz (15) in various plants.

FIG. 5.1–5.5—Electron micrographs of spinach chloroplasts. In Figures 5.1–5.4, the chloroplasts were isolated from mature leaves into cold 0.05 *M* PO_4 buffer, pH 6.5 by differential centrifugation. A suspension of the chloroplast material was permitted to dry on the screens and then washed with water to remove salts. ¹

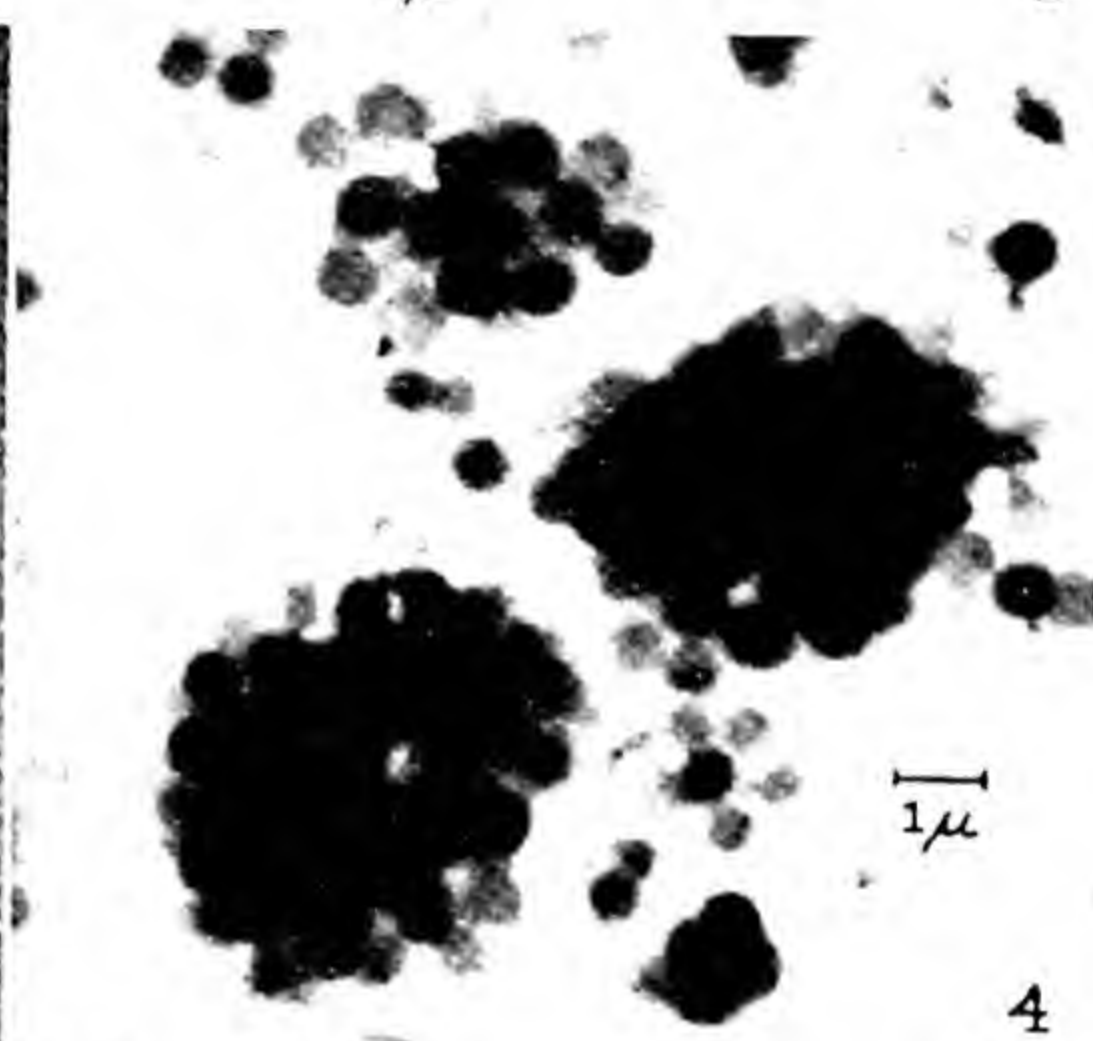
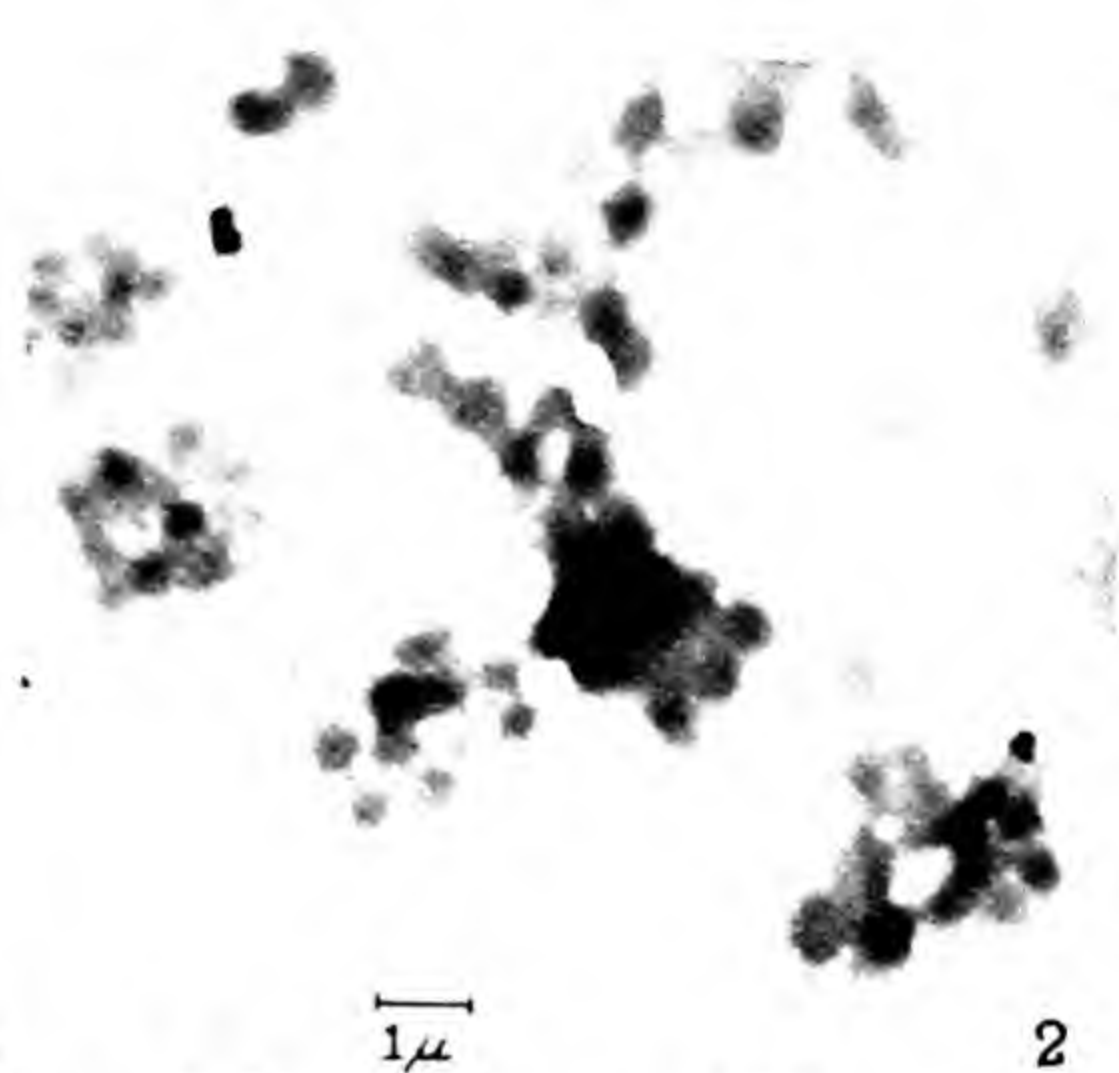
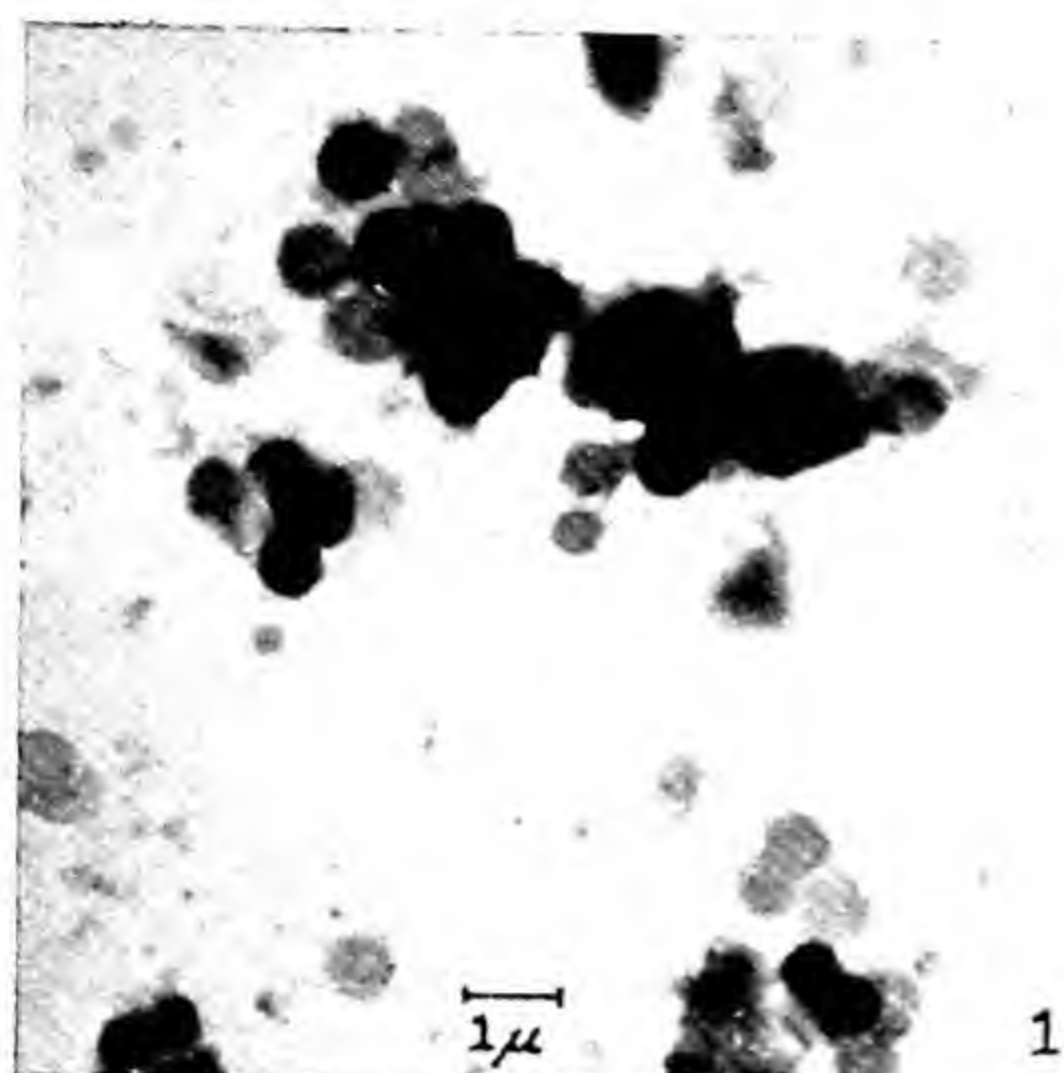
FIG. 5.1—Fragments of chloroplasts. The grana stand out as dense bodies.

FIG. 5.2—Same material as Figure 5.1, except that the chloroplast material on the screen was washed with absolute methanol. Such treatment presumably removes only fatty materials including the green and yellow pigments, leaving a proteinaceous framework of the grana. The grana are now seen to have diminished considerably in diameter and in density. (Figures 5.1 and 5.2 were taken on the same photographic plate.)

FIG. 5.3—A cluster of three grana shadowed with gold. The grana are held together by a film of matrix. No fine structure can be discerned. These grana have a diameter of about 6,000 Å and a thickness of about 800 Å as estimated from the shadowing angle and length of the shadow.

FIG. 5.4—A group of chloroplasts and chloroplast fragments showing the uniformity of the grana in a chloroplast and the variation in density of the individual grana in the same chloroplast.

FIG. 5.5—This material was isolated into 0.1 *M* NaCl at room temperature and rapidly mounted and dried on the screen. The speckled appearance is due to salt crystals. The large blebs and delicate foamy appearance of the chloroplast matrix are visible. Because this material has not been washed, one may observe the denser matrix background which makes it difficult to distinguish the individual grana.



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Geitler (6) reported them in some green algae, and suggested that under certain conditions grana were also visible in certain species of the blue-green algae as spherical bodies more highly refractile and more strongly colored than the surrounding plasm. The apparent absence of grana in many intact chloroplasts and their appearance on injury have deterred the acceptance of the grana as morphological units.

The grana are generally, and probably correctly, considered to contain the green and yellow pigments, although on this point there is room for more convincing experimental evidence. Recently Jungers and Doutreligne (16) studied the amyloplasts of potato, which turn green on exposure to light. The amyloplast covering a portion of the starch grain turns green, and at the edges a single layer of grana is observed. They report that here the individual granum can be seen to be green and the matrix colorless against the white background of the starch grain.

It may be estimated that there are several million chlorophyll molecules in a single granum. On the basis of a number of assumptions, Frey-Wyssling (11) proposed the hypothesis that chlorophyll might be distributed in some twenty to thirty parallel fatty layers in a granum; each fatty layer would be 50 Å thick, and one layer would be separated from another by aqueous protein layers some 250 Å in thickness. Although studies with polarized light reveal a low degree of orientation of the lipids of the chloroplast, this hypothesis should also lead to the expectation that oriented chlorophyll molecules should show dichroism. No dichroism has been found (17), suggesting that the chlorophyll molecules must have a more random distribution in the grana. Chlorophyll in the chloroplasts shows an absorption maximum at 6,810 Å. Also, its fluorescence is very low. These two facts suggest that chlorophyll might be in an aggregated (polymeric) state rather than mono-dispersed.

Electron microscope studies of mature spinach chloroplasts (18, 52) reveal the grana clearly, and confirm the idea that they are morphological units. Some forty to sixty grana are contained in a spinach chloroplast; when vacuum-dried, they are revealed as dense wafer-shaped bodies some 6,000 Å in diameter and 800 Å thick (Fig. 5.3), embedded in a protein-containing matrix. The grana in individual chloroplasts appear to be rather uniform in size, but may vary somewhat from one chloroplast to another (Fig. 5.4). In general, the density of the grana is quite high but some grana are observed to have a lesser density than most. When the grana are extracted with methanol, a residue probably of protein remains, and appears to make up less than half of the original material (Figs. 5.1 and 5.2) (18).

It is probable that in the uninjured chloroplast, the matrix

normally has a high density and a refractive index similar to that of the grana. When the matrix takes up water, swelling and vacuolating, the grana are spread apart and the refractive index of the matrix is lowered sufficiently to bring about the visibility of the grana. Whether the edges of the grana also round up somewhat is not known. As yet it has been impossible to demonstrate the swelling of the individual grana during vacuolation of the matrix, nor has the foamy structure of the chloroplasts, produced on injury, been adequately examined in the electron microscope.

Fragments of the chloroplasts containing grana and matrix still function in the Hill reaction for releasing O_2 . It will be important to determine whether the grana alone can function, or whether the matrix also will have to be considered in interpretations of the steps of the Hill reaction. At any rate, intact chloroplasts are not essential, at least not for the first stages of photosynthesis.

In connection with the electron microscope studies of chloroplast structure it should be interesting to follow the development of the grana in size and number as the plastids develop, and to study the structure of mutant plastids. Studies should also be continued into the finer structural details of the chloroplasts if the picture of photosynthesis is to be clearly understood.

Studies with the polarizing microscope do not reveal any specific orientations of the chlorophyll molecules in the plastid since no dichroism can be seen (17), contrary to earlier reports. In *Mougeotia*, *Closterium*, and other algae, the chloroplast is uniaxially negative in relation to the thickness of the plastid, indicating that the chloroplast as a unit may be uniaxially negative, or that the individual grana themselves may be (11). Using imbibition methods on osmium-fixed chloroplasts, Menke (19) interprets his findings as suggesting that the negative double refraction is caused by form birefringence, and that the chloroplast has a positive intrinsic birefringence, possibly due to the parallel arrangement of anisotropic lipid components as lamellar units. More recently A. Frey Wyssling and E. Steinmann (53) have concluded that the birefringence of the *Mougeotia* chloroplast is principally a "form" birefringence due essentially to protein layers interleaved with rather imperfectly oriented lipid molecules. The difficulty of interpreting the effects of fixation and other changes that might occur in imbibition procedures makes further study desirable. As yet, there is no evidence from electron microscope studies for the existence of lamellar units.

ORIGIN AND INHERITANCE OF CHLOROPLASTS

The chloroplasts are of interest not only because they represent the primary seat of photosynthesis, but also because they represent a unit of cytoplasm, which like the nucleus can be shown, in favor-

able examples, to be self-reproducing. In general, the evidence for the self-reproducing ability of the chloroplast is fourfold. (a) the chloroplasts may be observed to divide, and their continuity can be established from one cell to the next by direct observation; (b) the transmission of the chloroplasts through the cytoplasm of only one parent demonstrates continuity of the chloroplasts and inheritance in a non-Mendelian fashion; (c) the chloroplasts, once lost from a cell, cannot be generated again by that cell; (d) if a plastid mutates, such a mutation is inherited in a non-Mendelian fashion.

The division and continuity of the chloroplasts may be observed directly in the cells of the lower plants, especially in those plants which contain only one or a few chloroplasts per cell. In the algae, the division of the chloroplast may occur at an earlier or somewhat later stage than the division of the nucleus. The pyrenoids frequently divide at the same time, but in many forms they disappear during cell division to reappear in each daughter cell. In bryophytes and pteridophytes, plastids and mitochondria have been reported to exist side by side throughout the life cycle, neither of them losing their individuality.

In the angiosperms the continuity of the chloroplasts cannot be observed directly, since the "proplastids" cannot be distinguished from mitochondrial bodies and perhaps other kinds of granules, which are present in the very young vegetative cells. It is possible that problems of this type may be resolved by cytological studies with the electron microscope. For example, K. R. Porter, in unpublished studies on animal cells, has frequently observed the apparent origin of mitochondria from extremely small granular precursors.

In a classic study of the origin of the chloroplasts in the young mesophyll cells of the apical leaf bud meristem of maize developing in light, Randolph (20) found that granules 1μ or less in diameter were present, some 150 granules being shown per cell. The granules decreased in number as the cells enlarged. At the same time, some of the granules enlarged. When they had reached a size of 2μ or over, some of them appeared in pairs as if they had just divided. What factors govern the enlargement of some granules and not others is not known. When these granules were about $3-4\mu$ in diameter, pigment was just discernible and occasionally starch was observed in some of them. The well-developed seedling leaves had plastids $7-8\mu$ in diameter, a deep green in color. Granules also were present, indistinguishable from those that had originally enlarged into plastids. It is not yet known what factors are present to govern the total number of chloroplasts per cell.

The homology between plastids and mitochondria has been proposed by a number of investigators. Some evidence in favor of such a view is that the young chloroplasts and the mitochondria

have a number of properties in common. They behave similarly with respect to solvents and fixatives. For example, they are fixed by Zirkle's fixative (copper dichromate in 0.5 per cent pyridine) above pH 4.6–5.0 but not below that pH range. Mitochondria also behave osmotically in a manner similar to the chloroplasts, swelling and vesiculating in hypotonic solution, and shrinking and becoming slender in hypertonic media. Hogeboom *et al.* (21), for example, have recently shown that the mitochondria of liver cells can be isolated in their rod-shaped form if the cells are torn apart in 0.8 M sucrose solution.

Further support for the independent inheritance of the chloroplasts is gained from observations on the transmission of chloroplasts by way of maternal cytoplasm. In some of the algae one gamete, generally considered to be the male gamete, loses its chloroplast, and the new plant developing from the zygote bears only the chloroplasts that have been derived from the one originally present in the maternal cytoplasm. In 1890 Chmielevsky (3) reported observations of the spiral chloroplast of the "male" cell of *Rynchonema* sp. (Conjugatae) as it passed over into the "female" cell. The male chloroplast was seen to become yellow, thinner, and then to disintegrate into granules which later disappeared; only the female spiral filament remained green and gave rise to new chloroplasts. In *Bryopsis cupressoides* in which the microgametes and macrogametes each have single plastids, Oltmann, in 1904 (3), reported that on fusion of the gametes to form a zygote the smaller plastid of the microgamete disappeared. In some of the bryophytes and pteridophytes the motile male gamete loses most, if not all, of its cytoplasm with any contained plastids before it unites with the egg. Thus only the chloroplast of the egg is transmitted.

A classic example of chloroplast continuity in the angiosperms, demonstrated by maternal inheritance, is that described by Correns in 1909 (22). In *Mirabilis jalapa* var. *albomaculata* no functional plastids are transmitted from the pollen tube to the egg. Branches of this plant which bear only colorless plastids in their leaves can develop flowers and fruit. When flowers from such a branch are fertilized with pollen from a branch bearing leaves with green plastids, an offspring with colorless plastids is produced which soon dies. A reciprocal cross of flowers from a green branch with pollen from a white branch gives rise to an offspring with normal green plastids.

An interesting example and further demonstration of the self-duplicating activity of the chloroplast is one described by Lwoff and Dusi (23). In *Euglena mesnili* there are about 100 chloroplasts per cell and chlorophyll can be formed in the dark. When this species is cultured in the dark, multiplication of the cell, although slow, is

faster than the division of the chloroplasts. After some 15 months the cells contain only one or two green chloroplasts. Finally one may obtain individuals with no vestige of a chloroplast. These individuals are thenceforth incapable of giving rise to chloroplasts even in the light. Similarly, Van Wisselingh (24) reported that when *Spirogyra* cells were centrifuged and permitted to multiply, they would develop normally if they contained a nucleus and chloroplast, but cells which were formed containing a nucleus and only a small fragment of a chloroplast would die. Also of interest in this connection is the finding by L. Provasoli, S. H. Hutner, and A. Schatz (54) that streptomycin acting on *Euglena viridis* brings about the loss of all the chloroplasts. The organism remains colorless and devoid of chloroplasts throughout succeeding generations. Unlike the case reported by Lwoff however, the paramylon granules continue to be formed, and the orange eye spot, although smaller than normal, also remains.

Inheritance of a mutated plastid is further support for the self-duplication of the plastid. A study by Rhoades (25) of the iojap maize character of chlorophyll striping has revealed the following important observation. This character is brought about through the action of a gene which induces a change in the chloroplast. This change is permanent and remains even though the gene has been replaced. The mutated plastid, although induced by a nuclear factor, is thereafter transmitted independently of the nucleus.

FACTORS WHICH MODIFY THE CHEMICAL COMPOSITION OF THE CHLOROPLASTS

The composition of the chloroplast may be modified in a number of ways. It may be modified by genes, and these modifications will be inherited in a Mendelian way. It may be modified by changes in the chloroplasts themselves, and these changes will be inherited in a non-Mendelian way. Perhaps changes in other self-reproducing cytoplasmic units may modify the chloroplast, and these changes will also be inherited in a non-Mendelian way. The composition of the chloroplast will also be affected by factors that become apparent in the processes of cellular differentiation.

These chloroplast changes offer particularly valuable material for the study of the relation of structure to metabolic function and may prove of value in the analyses of various steps in the photosynthetic mechanism.

Gene alteration will bring about modification of the chloroplasts (26, 27). In maize, for example, at least sixty-five genes which can affect the formation and development of chloroplast pigments have been detected. Demerec (28) has listed thirteen genes which independently of each other produce pure albinos. Here the plastid

primordia develop at the same rate as do the normal, but at the time chlorophyll begins to appear in the normal plastid (i.e., at about 3μ diameter), the development in the albino stops and degeneration sets in. There are also several genes which produce yellow plastids, some twenty genes which produce delayed chlorophyll formation in the seedlings (virescent type), and some thirty genes which produce various types of pale green seedlings. In addition there are some thirty-five genes which produce variegated leaves; that is, where the normal and chlorophyll-deficient cells lie adjacent. The variegations may appear as longitudinal yellow or white stripes in the leaves, or as large blotches devoid of plastid pigments. The variegated leaves are gene-induced, but how the genes affect the development of the plastids is unknown. One suggestion for variegation (28) is the presence of an unstable gene which changes in one of the embryonic leaf cells to give rise to a change in the chloroplasts of the cells arising from this embryonic cell; or it may be due to a continually breaking chromosome with a high degree of chromosomal unbalance as revealed by McClintock (29) in the endosperm tissue of maize; or to other forms of unequal mitoses which might arise by somatic crossing over or reciprocal translocation (30).

Non-Mendelian chloroplast deficiencies may develop which are permanent and independent of the genic constitution. In contrast to such non-genic effects, the influence of a chromosomal gene on the expression of the plastid is generally only a temporary one, the expression of the plastid returning to normal once that gene is removed from the nuclear complement. The clearest demonstration of a non-Mendelian chloroplast deficiency is the one described by Rhoades. In the *iojap* character, the mutated plastid was induced by a genic factor, but when once induced, the mutated plastid was inherited independently of the nucleus. This result suggests that the nuclear gene had in some way brought about an irreversible destruction of a hereditary unit in the plastid.

Other hereditary units in the cytoplasm might conceivably affect the composition of the plastids although no experimental results are as yet at hand. Evidence from inter-species crosses (31) suggests that factors of the cytoplasm, apart from those of the plastids, are independently inheritable. The work of P. Michaelis on *Epilobium* clearly demonstrates that inheritable factors in the cytoplasm influence size of plant, size differences of leaves, flowers, etc. Even in combination with a homozygous foreign nucleus these cytoplasmic factors may remain relatively unchanged.

In addition to these hereditary modifications which may affect the chloroplast, one must consider the particular environment in

which the cells find themselves; e.g., factors influencing cellular differentiation, factors controlling the foodstuffs that get to particular cells, etc. The effect of cellular differentiation on the composition of the chloroplasts is seen in the epidermal cells of the leaf (with the exception of guard cells) and in the cells of roots; here the plastids which arise contain little or no chlorophyll. The general concept has developed in recent years that all self-duplicating bodies contain nucleoprotein material. Menke (32), and Woods and du Buy (33) have reported ribose nucleic acid in the mature chloroplasts. It would be of great interest to investigate the changes of nucleic acid content in relation to the development of these bodies.

CHEMICAL COMPOSITION OF THE CHLOROPLASTS

The chloroplasts are a complicated organization of chemical substances. As has been noted, they are controlled genetically by a large number of genes, and they also contain within themselves hereditary units which determine some of their characters. Besides hereditary units in the chloroplasts, one may imagine hereditary units present in the cytoplasm, which may act to influence the chloroplasts. In addition, cellular differentiation processes may affect the chloroplast, such as those that influence the plastids of roots, or of leaf epidermal cells, to remain essentially without chlorophyll even in the presence of light.

It is, therefore, not difficult to conceive that under certain conditions, one or several of the complex functions of the chloroplast may be lost or modified and certain functions may be enhanced. So, a loss of chlorophyll pigment, but not of yellow pigment, may occur (yellow mutant); or a decrease of the green, but not necessarily of the yellow pigment (pale-green mutant); or a loss of both pigments and an increase in protein formation (aleuroneplast); or a loss of both pigments and an increase in starch formation (amyloplast); or a loss of protein leaving carotene to crystallize (chromoplast of carrot root); or the pigments may be lost, and the production of protein and especially oil (eleaoplast) may be enhanced.

PROTEIN OF THE CHLOROPLASTS

The chloroplasts of some plants can be readily separated from the rest of the cytoplasm and nucleus. This is best accomplished by tearing apart the parenchyma cells of the leaf blade material in cold hypertonic (0.5 *M*) sucrose solution with the aid of a Waring blender, and subsequently separating the chloroplasts by differential centrifugation. Depending on the plant material, more or less intact chloroplasts can be obtained which can be subjected to analysis. By extracting the chloroplast pigments of the sample of isolated chloroplasts with acetone and comparing the intensity of this green

extract with a similarly prepared extract obtained from a unit weight of leaf, it is possible to relate the quantity of material in the chloroplasts to that in the leaf (34). For example, it is possible with such a procedure to find out what percentage of the total protein nitrogen of the leaf is present in the chloroplasts.

When the chloroplasts of mature leaves of tobacco and tomato were isolated and analyzed in the above manner, they were found to contain about 35–45 per cent of the protein-N of the parenchyma cell. Of the nitrogen of the chloroplasts some 13 per cent was soluble in alcohol-ether, approximately 7 per cent more was extracted with trichloroacetic acid, and the remainder, presumably protein-N, represented 80 per cent of the total-N of the chloroplasts (34).

For mature sudan grass Hanson, Barrien, and Wood (35) reported that 35–40 per cent of the total protein-N of the leaf was present in the isolated chloroplasts, and Galston (36) found about 30–40 per cent of the total leaf-N in the isolated chloroplasts of oat leaves. For spinach chloroplasts Menke (32) calculated that, on a dry weight basis, 44.8 per cent of the chloroplast was made up of protein, and on the average 35 per cent consisted of ether-soluble constituents.

Several studies have been made on the changes in protein nitrogen as the leaves and chloroplasts increase in size. Schulze and Schutz (37) observed that the protein-N of young leaves of *Acer negundo* increased as the leaves increased in size, attaining a maximum at the time the leaf attained its maximum size; then as the leaf aged, the content of protein decreased gradually. Similar observations were made on tobacco and tomato (34). Here an especially rapid increase in protein-N was noted when the leaf cells, after ceasing multiplication, were enlarging rapidly (i.e., from the time when the leaves were increasing from one-third of their fully expanded length up to the time when they were two-thirds of their maximum length). There was a tenfold increase in protein-N during this interval. From such data one may conclude that increase in cell size is not merely a thinning out of protoplasm and enlargement of the cell vacuole. There is actually a great increase in protoplasmic substance.

The chloroplasts have ceased most of their multiplication by the time the cells have ceased multiplying. At the stage where the leaf is two-thirds its maximum length the chloroplasts have enlarged considerably and were found to have increased approximately twelvefold in protein-N content (34). It is likely that this synthesis of protein takes place within the chloroplasts themselves. In general, the chloroplast protein increased or decreased in proportion to the

total leaf protein-N. In sudan grass the youngest leaves contained only 7 per cent of the total protein-N as chloroplast-N, but this rose to 30–40 per cent in the later stages (35).

Little can be said about the specific proteins making up the matrix and the grana of the chloroplast. That the proteins of the chloroplast as a whole are different from the other cytoplasmic proteins is suggested by the amino acid analyses of Chibnall (38) on spinach leaves. Lysine and histidine were 4.7 and 3.3 per cent of total protein-N in the chloroplasts and 6.2 and 2.2, respectively, in the rest of the cytoplasm, but there was essentially no difference in distribution of several other amino acid constituents; namely, arginine, tyrosine, tryptophane, cysteine, methionine, aspartic, and glutamic acid. In sudan grass Hanson *et al.* (35) found that the chloroplast protein contained about 70 per cent of the total protein sulfur of these leaves. According to these workers, the constancy of the ratio of chloroplast-N to chloroplast protein-S suggests that a single protein or a group of allied proteins is restricted to the chloroplast.

Of great interest in connection with chloroplasts is the question whether a specific protein-chlorophyll complex exists. On analogy with hemoglobin, which has four heme units per globin of molecular weight 66,000, there was postulated a chlorophyll protein complex of similar ratio; i.e., one chlorophyll per 17,000 mol. weight protein. Analysis of the isolated chloroplasts showed that there was much more chlorophyll present than could be accounted for on such a hypothesis (34). The ratio of chlorophyll to protein was found to be rather variable, depending on the species and the developmental stage of the leaf, values from 10 to 3 chlorophyll molecules per 17,000 mol. weight protein having been reported (8, 35). This ratio becomes even less meaningful if calculations are made on the assumption that all the green and yellow pigments are localized exclusively in the grana (8, 32). The following composition of a fraction termed the "granula" is reported by Warburg (39); chlorophyll, 9 per cent; ash, 3 per cent; P, 0.3 per cent; Fe, 0.1 per cent; Mn, .016 per cent; and Zn, 0.0068 per cent.

Other proteins in chloroplasts may be recognized by their enzymic activities. Spinach chloroplasts which had been extracted to remove the plastid pigments showed a pyridine hemochromogen spectrum indicating the presence of heme enzymes (40), including catalase (41). Li and Bonner (42) found that the oxidation of tannins in green leaves of tea, converting the tea to black leaf tea, was due to a polyphenol oxidase enzyme. This enzyme was present in the chloroplast, probably in the grana, but it was absent from the cytoplasm. A whole host of other enzymes must be present to bring

about the synthesis of the various proteins, carbohydrates, nucleic acids, pigments, etc., that are undoubtedly synthesized for the most part within the plastids themselves.

ETHER-SOLUBLE CONSTITUENTS OF THE CHLOROPLASTS

Analysis of the ether-soluble constituents of the chloroplasts, made on preparations of "chloroplast substance" (i.e., protoplasmic material of leaf cells that have been flocculated with calcium ions), indicated that this fraction made up some 30.7–39.5 per cent dry weight of the chloroplast substance in spinach, according to Menke (32), and 22.5–36.7 per cent according to Comar (43); for red clover Neish (41) reported 21.8 per cent. The high lipid content of the chloroplasts and its low content in the rest of the cytoplasm was indicated by the analysis of Chibnall on spinach (38); the ratio of protein to lipid in the chloroplasts was 39.6:25.1, and in the remaining cytoplasm was 96.5:1.9. According to Menke and Jacob (55), of the lipids, 50 per cent were fats, 20 per cent were sterols, 16 per cent were raw wax, and 2–7 per cent were phosphatides.

According to Comar (43), the lipid fraction of the chloroplast substance of spinach contained about 11.2 per cent of the total-N of this material, of which only about one-third was due to the chlorophylls. There remained then some 7–8 per cent of the total-N of the chloroplast substance to be accounted for, and it would be interesting to know whether such a fraction might represent phosphatides. In tomato chloroplasts 13 per cent of the total-N was found in the lipid fraction, of which some three-fourths was accounted for as chlorophyll-N (34). In mature tomato leaf blades, the lipid-P of the chloroplasts made up about 35–45 per cent of the total-P of the chloroplasts and about 35–65 per cent of the lipid-P of the leaf blade (40). In the runner bean Jordon and Chibnall (44) observed that the embryo axis contained only the magnesium salt of phosphatidic acid, which increased in amount during germination until the development of the prophylls, when a rapid change over to the Ca salt took place, the fat-soluble-Mg possibly being utilized in the synthesis of chlorophyll. J. H. C. Smith (45) investigated the ether-soluble-Mg of etiolated barley seedlings and found it to increase in the early stages of illumination, and to be higher in content than the Mg required for chlorophyll synthesis. After the initial stages of greening, the ether-soluble-Mg was only slightly in excess of the Mg in chlorophyll. He also noted the interesting correlation that albino corn seedlings contained only very small amounts of ether-soluble-Mg, but that in green corn seedlings the ether-soluble-Mg was proportional to the chlorophyll-Mg.

The chlorophylls are discussed in the succeeding pages. Obviously chlorophyll is an important fraction of the functioning

chloroplast. Chlorophyll in the intact leaf fluoresces very slightly. It possesses an absorption maximum at around 6,810 Å. Isolated chloroplasts have been obtained whose absorption maxima were at 6,790 Å, and the shift to shorter wave lengths was the greater the more damaged the chloroplasts appeared. These facts are yet to be explained satisfactorily.

The carotenoids also are discussed elsewhere. These pigments not only accompany the chlorophylls but appear to precede them in the development of the young plastids. The data of Frank (46) on the photo-induction of chlorophyll in etiolated *Avena coleoptiles* suggest that the carotenoids may be present behind the layers of protochlorophyll in the young plastids, since the carotenoids do not appear to screen off the light used for the photo-induction of chlorophyll synthesis. It would be interesting to know whether a similar arrangement of carotenoids lying behind the chlorophylls is present in the mature chloroplasts. In the young plastids of etiolated barley seedlings (47) large quantities of a number of carotenols are already present in a ratio similar to the ratios found in older green plastids of mature leaves. When seedlings are exposed to light, the carotenoids increase in amount, more or less keeping pace with the increase in protein and chlorophyll of the chloroplasts. In the mature leaves of a number of higher plants the weight ratio of chlorophylls:carotenoids is 2.4–3.0:1, and the weight ratio of carotenols:carotenes (i.e., principally lutein and carotene) is 4–6:1 (8). In terms of molecules, the approximate ratios of chlorophyll *a*: *b*: carotene: lutein would be as 6:2:1:3.

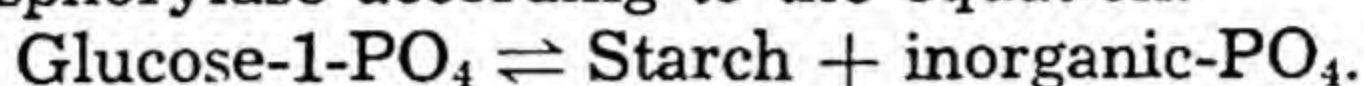
The yellow fall leaves lose much of their carotenols with the exception of zeaxanthin ($C_{40}H_{56}O_2$). Yellow portions of variegated leaves resemble fall leaves with respect to the carotenols. When leaves, especially of etiolated seedlings, are killed so as not to destroy oxidative enzymes, i.e., by anaesthetics or freezing and thawing, the carotenoid pigments rapidly disappear (47). Mobius (48) described the changes in autumn coloring of the foliage, finding the mesophyll cells to be filled with a watery sap of yellow refracting drops which had absorbed the yellow pigments of the disintegrated plastids. In the fall leaves of *Ginkgo biloba* and *Fagus sylvatica* the yellow pigments were contained in oil or in the watery sap.

CARBOHYDRATE STORAGE PRODUCTS IN THE CHLOROPLASTS

The chemical structure of starch and the mechanism for its biosynthesis have been established only very recently, and are summarized in a comprehensive review by Hassid (49). During rapid photosynthesis starch grains may be formed in the chloroplasts of the leaf mesophyll cells if the rate of carbohydrate formation exceeds the rate of its removal from the cells. The starch appears

to be formed in specific regions or vacuoles in the chloroplasts, these regions evidently being the sites for the localization of the enzyme phosphorylase. Two kinds of starch molecules are generally present in the starch grain; the minor component is β -amylose and the major component is α -amylose, which stains red with iodine. Waxy maize and waxy sorghum consist almost entirely of α -amylose.

The synthesis of starch from glucose-1- PO_4 is catalyzed by the enzyme phosphorylase according to the equation:



This equilibrium state is defined by the ratio: inorganic-P/ester-P. The acidity affects the equilibrium so that this ratio is 3.1 at pH 7.0 and 10.8 at pH 5.0. These data suggest that there would be a greater tendency toward starch formation if the chloroplasts were on the acid side of neutrality. Oxidative reactions which bring about the resynthesis of adenosine triphosphate from adenosine diphosphate + inorganic- PO_4 might be present, tending to remove inorganic- PO_4 from the neighborhood of the phosphorylase enzyme, thus favoring more starch formation. If glucose-1- PO_4 were present in relatively higher concentration, the tendency to greater starch formation would also be favored.

Starch may be hydrolyzed by amylase, especially during seed germination when the amylase activity is relatively high. But amylase activity in leaves appears to be a negligible factor in hydrolysis. In many leaves the starch content decreases rapidly as the leaves wilt, suggesting that here conditions are favorable for the conversion of starch to the glucose-1- PO_4 . Leaves killed by chloroform or frozen and then kept sterile for a month at 30° show no decrease in starch (47), indicating that such treatments readily destroy phosphorylase activity in the chloroplasts. Such treatment would not have inactivated amylase.

VITAMINS IN THE CHLOROPLASTS

Besides the chlorophyll molecules which bear the phytol chain, two other substances, vitamins K and E, are also present in the chloroplasts and also contain phytol as part of their structure. Studies of Dam and co-workers (50) have shown that vitamin K develops as the chloroplasts enlarge, but vitamin K formation is not necessarily dependent on complete formation of the normal chloroplasts. For example, the content of vitamin K is approximately the same in the pale green and in the normal green leaves of *Sambucus*. It is low in pea seedlings grown in the dark, but is synthesized in the light. Seedlings of *Picea canadensis*, however, which form chlorophyll in the dark, also form vitamin K in the dark. Spinach leaves contain approximately 40 μg . vitamin K per gram dry weight. When

leaves wither in the fall the vitamin K disappears only slowly. Vitamin E is localized for the most part in the chloroplasts where it is present to the extent of 0.08 per cent on a dry weight basis; in the cytoplasm there is only 0.002 per cent. Vitamin E also appears to develop in regions where photosynthesis does not occur, especially in connection with the formation of oils as in wheat germ, etc. Another chloroplast constituent, carotene, is the provitamin A, i.e., a precursor which is split in the liver to vitamin A.

Leaves, in general, have a higher ascorbic acid (vitamin C) content than other parts of plants, except meristematic tissues. The leaf attains its maximal content of vitamin C just before flowering. Giroud suggested that the blackening of chloroplasts with silver nitrate, first observed by Molisch, was due to the presence of ascorbic acid in these bodies. Weier (51) found the silver nitrate in the cell was reduced to elemental silver at a pH of 7, or above, but very slowly below pH 4, tending to corroborate the idea that this reducing substance was ascorbic acid. The reducing ability of the cells was lost very rapidly if they were killed by anaesthetics in the presence of oxygen, but if killed in an atmosphere free from oxygen, or by procedures which inactivated the oxidative enzymes, the reducing substance was still preserved. Although the silver reduction appears to be greatest in or on the chloroplasts it cannot be assumed as proven that the ascorbic acid is present exclusively in the chloroplasts.

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6.

Functions and Properties of the Chloroplast Pigments

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GREEN and yellow pigments of the chloroplasts play several basic roles in the metabolism of plants. Within the living plant cells and with the aid of absorbed sunlight, these chloroplast pigments initiate the complex metabolic reactions that are essential to the growth and development of autotrophic organisms. Because nearly every living thing depends upon green plants for food, this activity of the pigments is essential to the maintenance of virtually all life. From this point of view, chloroplast pigments are essential parts of the delicate organizational machinery of the organic world (107, 127).¹

PIGMENTS AND METABOLISM

The production of plant substance which results from the absorption of light by chloroplast pigments is always associated with the assimilation of water and carbon dioxide and usually with the liberation of oxygen. Through this involved photosynthesis of organic matter from inorganic substances, the pigments of the chloroplasts are geared to the cycle of the chemical elements. They form one of the connecting links between the inorganic and the organic realms.

In most autotrophic plants, pigments of the chloroplasts are indispensable to the photosynthetic processes, not to the catabolic metabolism. Marked deficiencies of the pigments are always associated with retardation of photosynthesis, but these pigment-deficient organisms often thrive and reproduce on organic food from other sources. For example, in nature, all colorless variants of green

¹To facilitate printing, authors' names, employed in the manuscript as a key to the bibliography, have been deleted by the editors and numbers substituted.

species are heterotrophic. Many colorless algae, including certain plastid-free species, are saprophytic; a few pigment-deficient algae and some pallid higher plants exist as parasites; and albino and yellow plant chimeras survive only as parts of green plants (5, 105, 120). (Albino and yellow seedlings that normally die as soon as the food reserves of the seed have been depleted grow to maturity when supplied with organic foods (53, 128).

But the normal complement of green and yellow pigments is not always evidence of photosynthetic capacity. A few green seedlings also die as soon as the food reserves of the endosperm have been exhausted (21). Presumably these plants are deficient in essential, colorless components of the photosynthetic apparatus).

PIGMENT FORMATION

In many autotrophic organisms, the formation of the green and yellow chloroplast pigments involves photochemical reactions apart from the production of organic matter by photosynthesis. Some of these photoreactions are coupled with extremely intricate metabolic processes (18). Thus in seed plants, chlorophyll formation depends upon exposure of the plants to light and to a liberal supply of oxygen as well. Other conditions that interfere with the metabolism of the plants also retard the formation of the pigments (62).

PHOTOTROPIC RESPONSES

Phototropisms and the growth habits of many plants depend upon photochemical processes which result from the absorption of light by colored substances. The bending of sprouts, shoots, and seedlings toward light, the height of plants, the distance between internodes, and sturdiness depend, to a large degree, upon the intensity and the spectral quality of the incident radiation and upon the spectral absorption properties of the pigments contained within the cells. Inhibition of the elongation of the first internode of oat seedlings by red light has been attributed to the preferential absorption of this light by the green pigments chlorophyll and protochlorophyll (46, 66). Other phototropisms in these seedlings result from absorption of light by the yellow pigments (63).

In some plants, flowering and the setting of fruits depend upon a delicate balance between the periods of illumination and of darkness. Respiration (163) and even the germination of some seeds are stimulated by light (37). Most algal gametes and many motile algae migrate toward the light, this phototropic response being controlled by special light receptors (120, 121) which contain pigments chemically related to the yellow constituents of the chloroplasts (122, 154). Some of these yellow pigments also affect the copulation and the sexuality of certain algal gametes (123).

All these phototropisms and many related phenomena involve small changes of organic materials relative to the natural, photochemical production of organic matter (44). Organisms that contain insufficient quantities of pigments to provide their own nourishment by photosynthesis exhibit normal phototropic responses and growth habits. Despite the small amounts of materials that must undergo chemical change, these extremely sensitive phototropisms are indispensable to the survival of most plants.

PIGMENTS AND ANIMAL METABOLISM

Probably because of the widespread occurrence of chloroplast pigments in the vegetable food upon which all animals depend, some of these colored compounds have been utilized as key substances in many metabolic processes. Notable examples are: the vitamin A activity of certain yellow, carotenoid materials derived from chloroplasts; the photochemical activity of carotenoids in the dark-adapted eye and in the stigma of algae and of algal gametes; and the enzymatic and oxygen-carrying properties of numerous tetrapyrrolic compounds related to chlorophyll. Most of the yellow markings of birds and fishes that are presumed to serve as protective coloration and that undoubtedly influenced the evolution of many species are due to pigments obtained from the chloroplasts of the vegetable food (38).

THE COURSE OF RESEARCH

Recognition of the importance of colored substances in so many critical aspects of plant and animal physiology has stimulated numerous investigations along many lines with the hope that clues to the mechanism of the basic chemical and photosynthetic reactions might be discovered. Most of these studies fall into two principal categories: physical and chemical properties of the colored materials extracted from the organisms; and properties and reactions of these substances within the living cells.

Studies of the extracts of plants have yielded a large body of information concerning the number and kinds of pigments, their chemical and physical properties, their molecular structures, and their natural distribution. These facts, in turn, have provided a basis for determination of the photosynthetic activity of the individual, colored constituents of the chloroplasts. Information regarding the occurrence of particular pigments in plants of different taxonomic groups has revealed unsuspected phylogenetic relationships among autotrophic organisms, and it has given indications of the age of the photosynthetic apparatus.

Studies of pigments within living organisms have been hindered by the lability of the reactive systems and by the extreme insolubility of the colored substances in the aqueous media comprising the

plant and animal tissues. Once the cells have been killed, the pigments undergo a variety of oxidative and hydrolytic reactions which are not observed in the plastids of living plants. Pigments extracted from the plastids of one organism can not be introduced into the functional plastids of another.

With few exceptions, discoveries of physiological processes stimulated by light have proceeded faster than the interpretation of these phenomena in relation to the physical and chemical properties of the pigments. As a consequence, a great gap has developed between physiological investigations on living organisms and conventional physical and chemical studies of the extracted pigments.

RESEARCH FRONTIERS

Much scientific prospecting must yet be done in order to unearth the most significant facts concerning the chloroplast pigments and their reactions. There is no reason to believe that all these substances have been discovered. Many have been but partially described. The chemical structure of most of them remains undetermined. Most of the natural synthetic reactions that lead to their formation are obscure. Their condition and arrangement within the plastids are not known. Physiological and genetic control of the formation of different colored substances in unrelated species has yet to be elucidated. The mechanism of the transfer of absorbed energy from the pigments to other substances is a major unsolved problem.

The trails that lead into these uncharted regions are difficult to follow, for the signposts have been lettered in the language of specialists. Rather than grope along these indistinct pathways, the general reader may wish to stop here with the realization that chloroplast pigments play essential, complicated roles in plant and animal metabolism. Some readers with a flair for chemical detail may elect to peruse the following sections in which the sources, properties, and kinds of pigments are described. Those with a special interest in the mechanism of physiological processes, such as photosynthesis, might turn to the section dealing with the properties of the pigments within the chloroplasts. Perhaps a few may prefer to study the final section which concerns the age and the evolution of the photosynthetic apparatus.

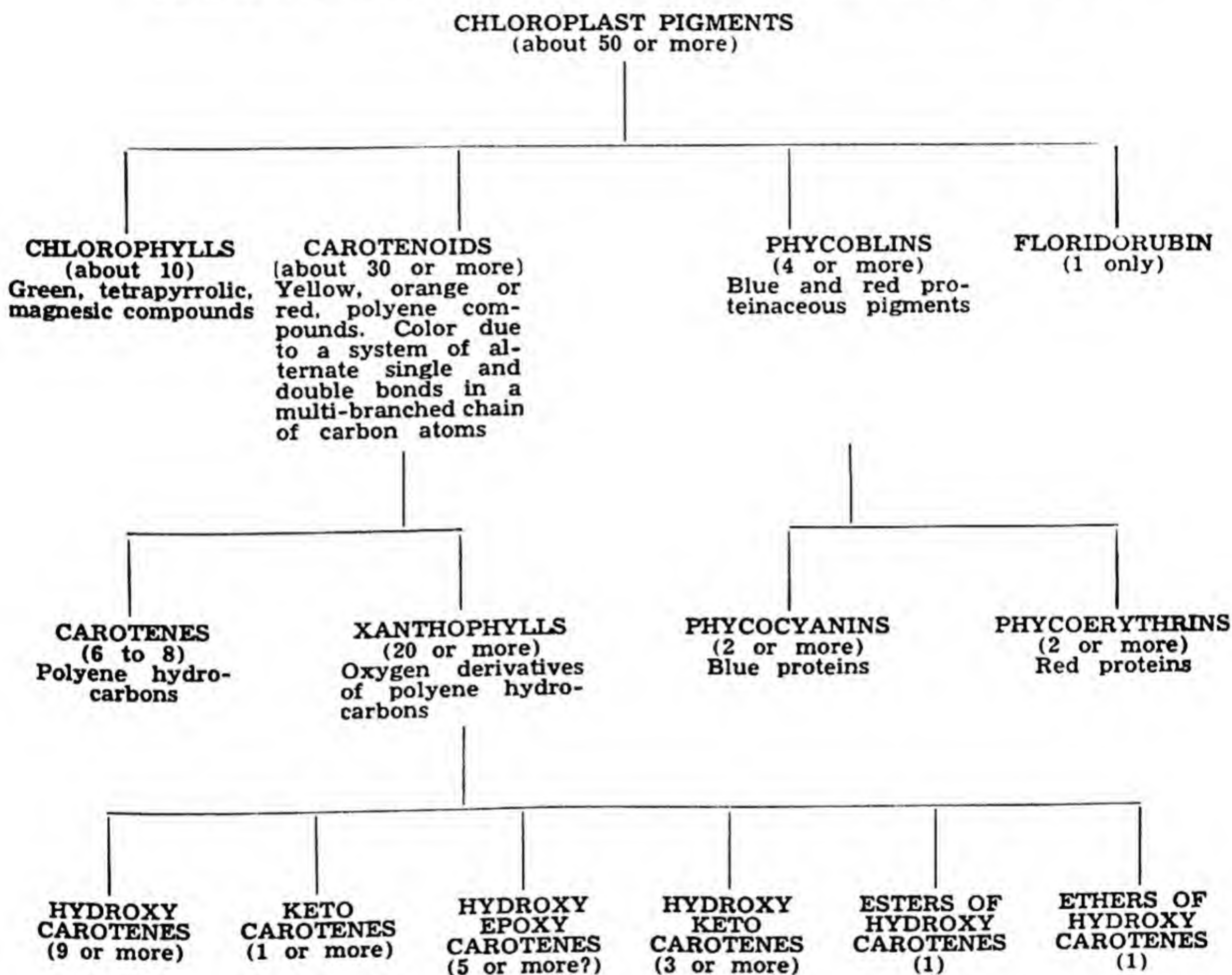
PRINCIPAL KINDS OF CHLOROPLAST PIGMENTS

FEW KINDS OF PIGMENTS

With respect to color and chemical properties, chloroplast pigments may be placed in three principal groups: chlorophylls, carotenoids, and phycobilins. On the basis of chemical structure, pigments in some of these groups can be segregated into smaller sub-

groups, as is illustrated in Table 6.1. The incompleteness of information regarding the molecular structure of many xanthophylls makes precise classification of them impossible. Many carotenoids in addition to the number indicated in this table have been isolated from plant structures other than the chloroplasts; for example, the

TABLE 6.1
THE PRINCIPAL KINDS OF CHLOROPLAST PIGMENTS, THE APPROXIMATE NUMBER OF PIGMENTS OF EACH KIND, AND SOME DISTINCTIVE PROPERTIES OF THE PIGMENTS



yellow plastids of algal gametes, fruits, roots, flowers, etc. (89, 131, 169, 170).

Because of the great differences among the properties of some pigments and because of the slight differences among the properties of others, no single method of analysis serves for extraction, separation, identification, and classification of all these colored compounds. The proteinaceous phycobilins, which do not dissolve in organic solvents, are extracted from fresh plant material with water, either

by grinding fresh cells with abrasives and water (135) or by allowing killed cells to undergo autolysis in the presence of a preservative. But even after extensive disintegration and after prolonged autolysis, some of the red algae retain the bulk of their pigments in an insoluble condition. Soluble phycobilins in the aqueous extracts of algae are crystallized by addition of ammonium sulfate, as summarized in the review by Boresch and in the papers by Svedberg and his co-workers (12, 143, 144, 145).

Chlorophylls and carotenoids, being insoluble in water, are extracted from fresh plant material with an excess of methanol, ethanol, or acetone. Enzymatic and oxidative alteration of the pigments may be retarded by brief immersion of the fresh plant material in boiling water before extraction with the organic solvents (131). Green and yellow pigments may also be extracted from carefully dried green tissues with a variety of organic solvents (101, 169, 172).

Resolution of the mixtures of chlorophylls and carotenoids in the extracts of plants has been accomplished, in part, by partition of the pigments between immiscible solvents, such as aqueous methanol and petroleum ether (101) and to a very great degree, by adsorption in Tswett columns (34, 135, 171, 172). This latter chromatographic adsorption technique and spectral absorption methods have been widely utilized for detection and characterization of the pigments, especially in the exploratory investigations preliminary to the determination of chemical composition and molecular structure (141, 142).

DISTRIBUTION OF THE PRINCIPAL KINDS OF CHLOROPLAST PIGMENTS IN PLANTS

All autotrophic organisms ranging from the blue-green algae to the higher plants contain chlorophylls, carotenes, and xanthophylls. Red algae (Rhodophyta) and blue-green algae (Myxophyta) usually contain phycobilins in addition to the chlorophylls and carotenoids. Neither chlorophylls nor phycobilins have been found in the absence of carotenes and xanthophylls (137, 138). By contrast, carotenoids often occur in the absence or near absence of other pigments. In etiolated seedlings and in etiolated sprouts and shoots, formation of carotenoids precedes formation of the green pigments (42, 113); and in fruits and flowers carotenoids usually remain after disappearance of the chlorophylls (169, 170).

Purple bacteria and green bacteria, which thrive in the presence of organic matter or of hydrogen sulfide, and which combine photosynthetic and chemosynthetic processes for the utilization of carbon dioxide, also contain chlorophylls, carotenes, and xanthophylls (34,

103, 150, 151). None of these pigmented bacteria have been observed to liberate oxygen (151).

✓ AMOUNT OF CHLOROPLAST PIGMENTS DEPENDENCE UPON ENVIRONMENT

The total amount of the chloroplast pigments varies with the species, with the age of the organisms, with their genetic composition, and with the environment in which the plants are grown. Grown in different environments, a single species of higher plants or of green algae may vary in color from deep green to pale yellow, bronze, or red. A blue-green alga may vary from deep blue-green to green or yellow; a red alga from red to green, purple, or black. Grown in the same environment, unrelated species may yield very different amounts of the pigments, a striking example being the yellowing and bleaching action of direct sunlight upon shade plants, such as the African violet, *Saintpaulia ionantha*, contrasted to the greening action of direct sunlight upon most autotrophic plants. Usually, moderate temperatures, light of low or moderate intensity, and adequate supplies of mineral nutrients, particularly of sulfur, manganese, iron, zinc, potassium, and nitrogen, promote formation of the largest amounts of the pigments (14, 118, 119, 96). But the interrelationship of temperature, light intensity, mineral nutrition, and pigment formation is extremely complex. As an indication of this complexity, a few seedlings and many algae form chlorophyll in the dark (96). Retention of chlorophyll by *Euglena* in the dark depends upon the organic nutrition of the organisms (100).

Well-nourished algae may contain an amount of the chloroplast pigments equivalent to nearly 2 per cent of the fresh weight of the green tissue or from 4 to nearly 8 per cent of the dry chlorophyllous tissue (138). In the chloroplasts, concentration of chlorophyll must be several times that of this pigment in the entire cells. Chloroplasts of higher plants contain nearly as much pigment as that found in the green algae, a field of investigation recently summarized by Rabinowitch (107).

Thus far, no specific functional importance has been attributed to small variations of the pigment content. Over considerable variation of pigment concentration, the rate of photosynthesis may remain nearly constant; hence, the rate at which each pigment molecule absorbs and transfers energy must vary a great deal (167).

Very low pigment content is invariably associated with a decrease in the yields of agricultural plants and with a decrease in photosynthetic activity (49). In pineapple leaves the amount of chloroplast pigments is proportional to the amount of protein and to the age of the leaves (118). In *Chlorella* low pigment content caused by

deficiency of nitrogen is associated with a very large yield of fat relative to protein and carbohydrate (129).

At first sight, variation of the pigment content of autotrophic organisms by use of different nutritional conditions might be expected to provide a means for investigation of the photosynthetic efficiency of the pigments, both in relation to their total amount and in respect to their relative amounts (27). Such studies are complicated by the fact that variation of the pigment content may be associated with variation of other important cell constituents (36); with variation in the composition of the organisms (51, 98, 118, 129); and, in some instances, with the production of toxic substances (104).

RELATIVE PROPORTIONS OF THE SEVERAL KINDS OF PIGMENTS

As one would infer from the green color of autotrophic plants, chlorophylls are the most abundant chloroplast pigments. In green vegetative tissue, chlorophylls usually comprise about two-thirds or more of the total amount of coloring matter; xanthophylls represent about four-fifteenths, and carotenes, about one-fifteenth. These proportions vary within a plant, with the species, and with the environment (for a summary, see 107, p. 413; 138).

Relative amounts of the several kinds of chloroplast pigments are usually fairly constant in different parts of a given plant as long as the cells are growing rapidly. In old leaves, in variegated regions, in sun leaves, and in portions of algal thalli bearing gametes, the proportions of the chlorophylls tend to decrease relative to the carotenoids. This effect is demonstrated each year as the autumn colors appear at the onset of cold weather, and it is shown by chlorotic plants, by ripening fruits, by maturing flowers, and by certain algae lacking water or nitrogen (*Trentepohlia* sp.) or exposed to high salt concentration (*Dunaliella* sp.) (39). These extreme variations in the proportions of the pigments are usually associated with a decrease in growth rate or photosynthetic capacity.

Chloroplasts of many plants (Chrysophyta, Pyrrophyta, and Phaeophyta) appear yellow or brown rather than green. This yellow color of the plastids seems to result from the physical state or condition of the pigments or from their geometric arrangement rather than from a preponderance of the yellow pigments. Exposure of these plants to heat causes the plastids to turn green. Extraction of the heated plants or of the fresh plants with alcohol yields green solutions in which chlorophylls predominate (141).

Amounts of the phycobilins in red and in blue-green algae vary a great deal from species to species. The color of *Halosaccion glandi-*

forme, a red alga, so closely resembles that of the common species of green algae that this organism was first reported as an *Ulva* (*U. glandiformis*). Many red algae (*Constantinea simplex* and *Erythrophyllum delesseriodes*) contain so much phycoerythrin that it obscures or masks the green and yellow pigments. As all these species grow near the mean low-tide level, the different proportions of phycoerythrin can scarcely be attributed to spectral differences of the incident light.

Similar variations in the color of blue-green algae, of diatoms, of green algae, and of higher plants occur under natural conditions. Even greater variations have been produced by restriction of the mineral nutrients in the culture medium and by variation of the intensity and spectral quality of the incident radiation (4, 10, 12, 23, 50, 94, 121). Except in a few extreme cases where growth usually ceases, variation of the color of chloroplasts in response to variation in the environment usually involves changes in the proportions of the pigments rather than production of new, or uncommon, colored substances.

In most instances, these variations in color may result from variation of several different conditions. For one set of conditions, one factor, as light intensity, may determine the color change; for slightly different conditions, another factor, as temperature or salt concentration, may determine the color change. Variation of pigment composition with changes in the genetic make-up of the plants (21) may indicate that the synthetic capacities of the plant have been lowered for the usual environmental conditions, not that certain genes have been eliminated as proposed by Beadle (8). The concept of limiting factors, applied so profitably to the study of photosynthesis (127), might be extended with equal success to studies of pigment formation in various organisms.

INDIVIDUAL CHLOROPLAST PIGMENTS DISTRIBUTION OF PARTICULAR CHLOROPLAST PIGMENTS IN AUTOTROPHIC PLANTS

Autotrophic plants and chemo-autotrophic bacteria each contain one or two chlorophylls, from one to three carotenes, and from two or three to six or more xanthophylls. In addition to these pigments most red algae and most blue-green algae contain one or two phyco-bilins.

The occurrence of particular chloroplast pigments in the plant kingdom follows a definite pattern. Some of the pigments are confined to plants of one or two taxonomic groups; other pigments occur in plants of several groups; a few pigments occur in most plants;

TABLE 6.2

PRINCIPAL PIGMENTS AND PHOTOSYNTHETIC PRODUCTS OF VARIOUS KINDS OF PLANTS. ASSEMBLED FROM (137, 138, 120, 121)

Plants	Chlorophylls	Carotenes	Principal Xanthophylls	Cell Walls	Storage Products	Inorganic Products
Seed plants	$a + b$	$\beta \neq \alpha$	Lutein, Neoxanthin, Violaxanthin	Cellulose, Pectose	Starch (Fat)	O ₂
Ferns	$a + b$	$\beta \neq \alpha$	Lutein, Neoxanthin, Violaxanthin	Cellulose, Pectose	Starch (Fat)	O ₂
Mosses	$a + b$	$\beta \neq \alpha$	Lutein, Neoxanthin, Violaxanthin	Cellulose, Pectose	Starch (Fat)	O ₂
Stoneworts	$a + b$	$\beta \neq \alpha$	Lutein, Neoxanthin, Violaxanthin	Cellulose, Pectose	Starch (Fat)	O ₂
Green algae	$a + b$	$\beta \neq \alpha$	Lutein, Neoxanthin, Violaxanthin	Cellulose, Pectose	Starch (Fat)	O ₂
(except Siphonales)						
Green algae	$a + b$	$\alpha \neq \beta$	Siphonocin, Siphonaxanthin, Lutein, Neoxanthin, Violaxanthin	Cellulose, Pectose	Starch (Fat)	O ₂
(Siphonales)						
Vaucheria	a	$\beta \neq \alpha$	Unnamed	Pectose, Cellulose	Fats	O ₂
Tribonema	$a \neq e$	$\beta \neq \alpha$	Identical with those of Vaucheria	Pectose	Leucosin Fat	O ₂
Euglena	$a + b$	$\beta \neq \alpha$	Unnamed	(Naked)	Paramylum Fat	O ₂
Red algae*	$a \neq d$	$\beta \neq \alpha$	Lutein, Zeaxanthin	Cellulose, Pectose	Florideen Starch	O ₂
Brown algae	$a + c$	$\beta \neq \alpha$	Fucoxanthin, Violaxanthin	Cellulose, Pectose	Floridoside Soluble sugars	O ₂
Diatoms	$a + c$	$\beta \neq \alpha$	Fucoxanthin, Diadinoxanthin, Diatoxanthin	Pectose (Silica)	Fat, Volutin	O ₂
Dinoflagellates	$a + c$	$\beta \neq \alpha$	Peridinin, Diadinoxanthin, Dinoxanthin	Cellulose (Pectose)	Starch	O ₂
Blue-green algae*	a	$\beta \neq \alpha$	Myxoxanthin, Myxoxanthophyll, Lutein, Zeaxanthin	Pectose, Cellulose	Fat Glycogen? Fat, Proteins	O ₂

* These plants also contain phycobilins in addition to the chlorophylls and carotenoids.

and one pigment, chlorophyll *a*, is common to all autotrophic organisms except the pigmented bacteria.

Under conditions that favor normal growth and development, the principal chloroplast pigments are remarkably constant for species of each major taxonomic group. Exceptions are found among the red algae and the blue-green algae with respect to the occurrence of the major phycobilins, which are absent from a few species (120), and with respect to minor phycobilins, which are not found in about half the species (81, 138, 143, 144, 145).

Detailed information regarding the occurrence of particular pigments in autotrophic plants of major taxonomic divisions is given in Table 6.2 along with some of the conspicuous structural materials and food products characteristic of the organisms. More detailed tables showing the pigments reported by various authors and the individual species examined have appeared elsewhere (137, 138).

When conditions are unfavorable for the normal development of autotrophic plants, the proportions of the pigments are subject to great variation. For example, when exposed to light of very high intensity, plants that normally contain large proportions of chlorophyll *b* relative to chlorophyll *a* (ratio *b* to *a* = 1:2) frequently lose chlorophyll *b* faster than chlorophyll *a*, so that the ratio of *b* to *a* may approximate 1:4 or 1:5 (114, 115). Leaves normally deficient in chlorophyll (*aurea* leaves) also contain much chlorophyll *a* relative to chlorophyll *b* (115). In bright light, chlorophylls usually decrease faster than carotenoids so that the ratio of chlorophylls to carotenoids decreases. In etiolated seedlings exposed to light, this ratio increases due to rapid formation of chlorophyll. The ratio of carotenes to xanthophylls also varies when etiolated seedlings are exposed to light, the variations of these proportions depending to some extent upon the plant (113, 132).

When certain plants are subjected to conditions that restrict their growth and development, additional pigments may appear in the chloroplasts. These new pigments are usually uncommon carotenoids which are not found in the normal green tissue. Their formation is concomitant with a decrease or disappearance of the chlorophylls. The red rhodoxanthin, which colors the older fronds of *Potamogeton* and the bronze winter needles of conifers, such as *Cryptomeria japonica*, has not been found in the young foliage of these or of any other plants (23, 82). Similarly, an uncommon ketonic carotenoid is formed in the green alga *Protosiphon* when the cultures have ceased rapid growth and have lost their chlorophyll (138). A similar pigment, possibly identical with astacene of crustaceans, occurs in red forms of *Hämatococcus* (149).

/ CHLOROPLAST PIGMENTS OF FRUITS AND FLOWERS

The striking changes of coloration that occur when the chlorophyll disappears as green fruits and flowers mature are often due to formation of special carotenoids not normally found in green parts of the same plant. Examples of such pigments and their sources are: lycopene from tomatoes; prolycopene from berries of *Arum orientale*; physalein from calyx of *Physalis alkekengi*; eschscholtz-xanthin from petals of the California poppy; and capsanthin from red peppers. Many additional examples may be found in monographs concerning the carotenoid pigments (89, 101, 131, 171).

Most of these special plastid pigments are formed in the dark as well as in light provided the plant has sufficient organic reserves. So far as is known, these special, colored constituents of the yellow plastids do not play essential metabolic roles, because pigment-free fruits and flowers have frequently been observed in nature, and they are easily obtained by selective breeding. Formation of the special pigments is not dependent upon the presence of chlorophyll in the tissue, as shown by appearance of lycopene in colorless tomatoes grown in black bags (126). Formation of these yellow plastid pigments is usually associated with an accumulation of fat globules in which the pigments may be dissolved, but fat accumulation is apparently independent of pigment synthesis. The effect of environmental conditions upon formation of these special carotenoid pigments and the possible roles of the pigments in the attraction of pollinating insects are beyond the scope of this report (101).

Many of the xanthophylls of fruits and flowers occur with their hydroxyl groups esterified with fatty acids. Xanthophylls of the chloroplasts rarely occur as esters, only one ester-like pigment, siphonein, having been reported in green organisms, green algae of the order Siphonales (138) (see Table 6.2).

Variation of chloroplast pigments after growth of the organisms has been retarded demonstrates a latent capacity of the cells for modification of the colored constituents. By contrast, the constancy of the pigments in rapidly growing green tissues points to some unrecognized link between photosynthesis and the specific pigment mixture common to plants of each taxonomic group.

/ PROPERTIES CHARACTERISTIC OF CHLOROPHYLLS
COLOR

As indicated by their name, chlorophylls are distinguished by their green color. They are usually detected by their spectral absorption properties and by their solubility in fat solvents. All chlorophylls exhibit two pronounced absorption bands, one in the blue or blue-green and one in the red or infrared region of the spectrum (see Fig. 6.1). Less pronounced absorption bands appear

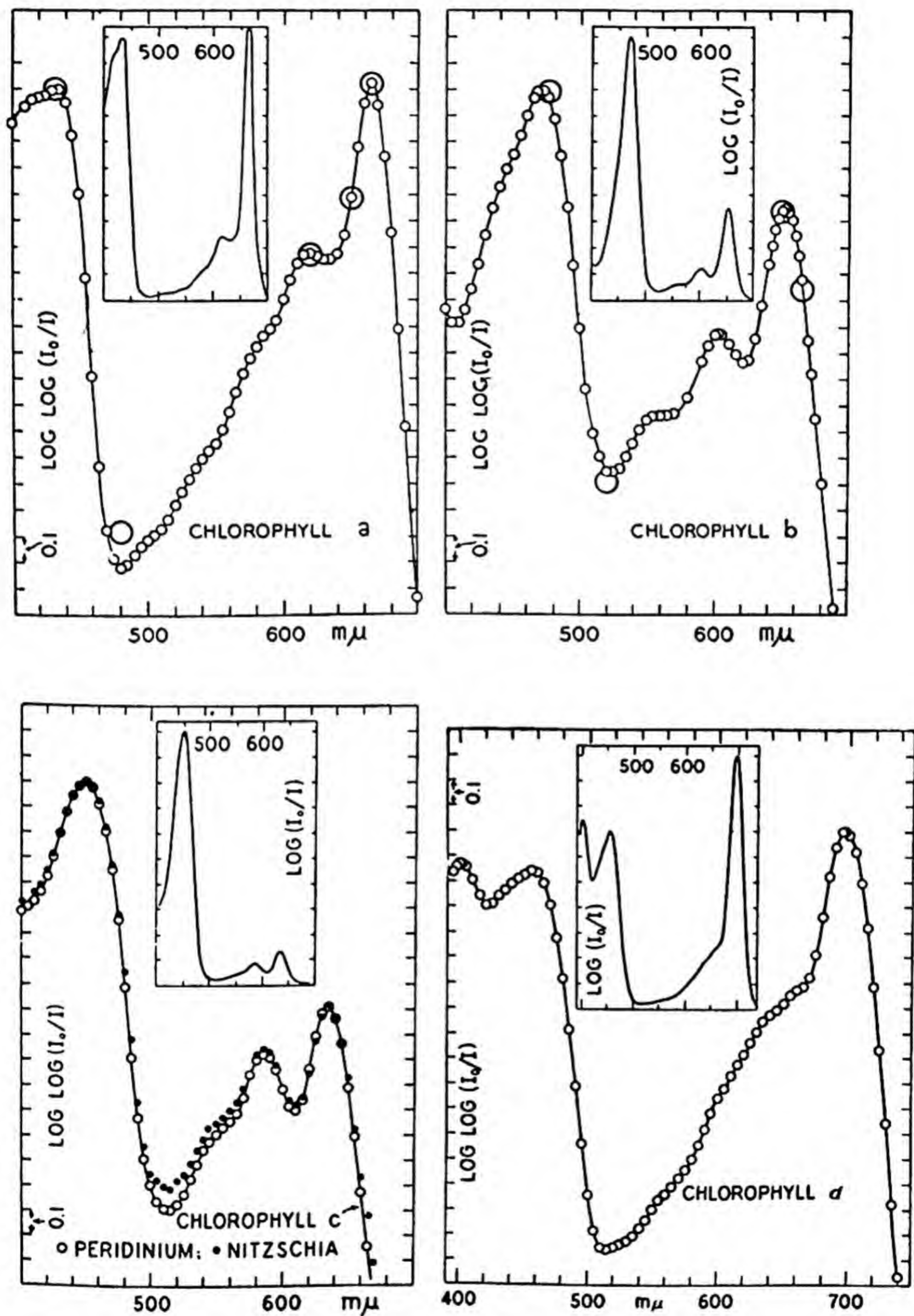


FIG. 6.1—Characteristic spectral absorption curves of several chlorophylls dissolved in methanol. The large circles represent values reported by Mackinney. Sources of curves are given under properties of the individual chlorophylls.

in the yellow and orange region of the spectrum. The absorption coefficients for green light are only about 3 to 5 per cent of those for blue and red light, depending to some extent upon the solvent, but relative to the weight of the pigment, the absorption in the green is appreciable (19, 85, 172, 173). A 10 per cent solution of chlorophyll *a* in a layer only 10 μ thick absorbs some 30 per cent of the incident green light and all of the incident blue and red light (138). As a result, very concentrated solutions of the chlorophylls may appear deep green or black; dilute solutions appear yellow-green. This effect accounts, in part, for the rapid transition of the color of plants from green to yellow as the chlorophyll decreases.

Wave lengths of the absorption maxima and the shapes of the spectral absorption curves of each pigment vary significantly with the solvent and with the condition of the pigment. Solid chlorophyll *a*, colloidal chlorophyll, and adsorbed chlorophyll exhibit spectral absorption maxima at longer wave lengths, 676 to 678 $m\mu$, than chlorophyll dissolved in any known solvent, 660 to 673 $m\mu$ (1, 16, 32, 35, 58, 76, 106).

Color in all the chlorophylls is due to a system of conjugated double bonds in a tetrapyrrolic (porphyrin) ring system. All reactions that affect this system of conjugated double bonds or that modify the porphyrin ring, affect the spectral absorption properties (34).

Solutions of the chlorophylls are strongly fluorescent and phosphorescent. Fluorescence maxima occur at wave lengths near those of the absorption maxima in the red region of the spectrum (22, 88); phosphorescence maxima occur at much longer wave lengths (beyond 800 $m\mu$) (17). Solid chlorophyll and chlorophyll in the plant are weakly fluorescent. Whether or not this difference in fluorescence is due to a lower fluorescence of the solid chlorophyll or to absorption of the fluorescent light by the more concentrated pigment is not clear from the experiments performed thus far (174).

✓ STRUCTURE

So far as is known, all chlorophylls contain magnesium at the center of the porphyrin ring, which forms the nucleus of the molecule. Two carboxyl groups combined in the form of a methyl and a phytyl ester are located on the periphery of the cyclic structure of all chlorophylls whose molecular structure has been determined (34, 111, 130).

✓ LABILITY

Chlorophylls are remarkably labile substances, each pigment being capable of existence in two or more isomeric forms. Living plants contain only one isomeric form of each pigment, but once

the pigments have been extracted, additional isomers are rapidly produced, detectable quantities appearing in about one hour at room temperature. When solutions of a chlorophyll are heated, equilibrium among the isomers may be established in a few minutes. Most of these artificial pigments exhibit spectral absorption properties similar to those of the natural isomers; hence they can not be detected by spectroscopic methods until after they have been separated from the mixtures by adsorption in Tswett columns (88, 139). The presence of these isomers in various chlorophyll preparations probably would not have had a very great effect upon the spectral absorption properties of the chlorophylls, which have been measured very carefully in a variety of solvents and which are cited explicitly in the following sections.

CHEMICAL REACTIONS

Extracted chlorophylls undergo a variety of oxidation, reduction, and hydrolytic reactions. Strong oxidizing agents (ferric ion) and active reducing agents (zinc powder) convert these green pigments into labile, colorless products that can be reconverted into green substances by reversal of the respective reaction, i.e., by reduction or by oxidation (108, 111); but the identity of these regenerated products with the natural chlorophylls has not been established with certainty (111).

At the dropping mercury electrode, chlorophylls are reduced with difficulty, requiring a potential nearly equivalent to that needed for reduction of carbon dioxide. This electrometric reduction of chlorophyll occurs in the unsaturated phytyl group rather than in the system of conjugated double bonds (153).

When leaves are killed with anesthetics or by grinding, oxidative and hydrolytic reactions contribute to the formation of some dozen or more green pigments that can be isolated by chromatographic adsorption methods. None of these alteration products has ever been observed in carefully prepared extracts of fresh plant material (141).

SOURCES AND PROPERTIES OF INDIVIDUAL CHLOROPHYLLS

Chlorophyll a

Principal sources: Green parts of all autotrophic plants except bacteria.

Formula: $C_{55}H_{72}O_5N_4Mg$.

Optical rotation: $[\alpha]_{720\text{ m}\mu}^{25^\circ} = -262^\circ$ (130).

Number of double bonds: 15 including 2 ester groups.

Principal absorption maxima ($m\mu$):

In ether: 430, 660 (172).

In methanol: 432.5, 665 (85).

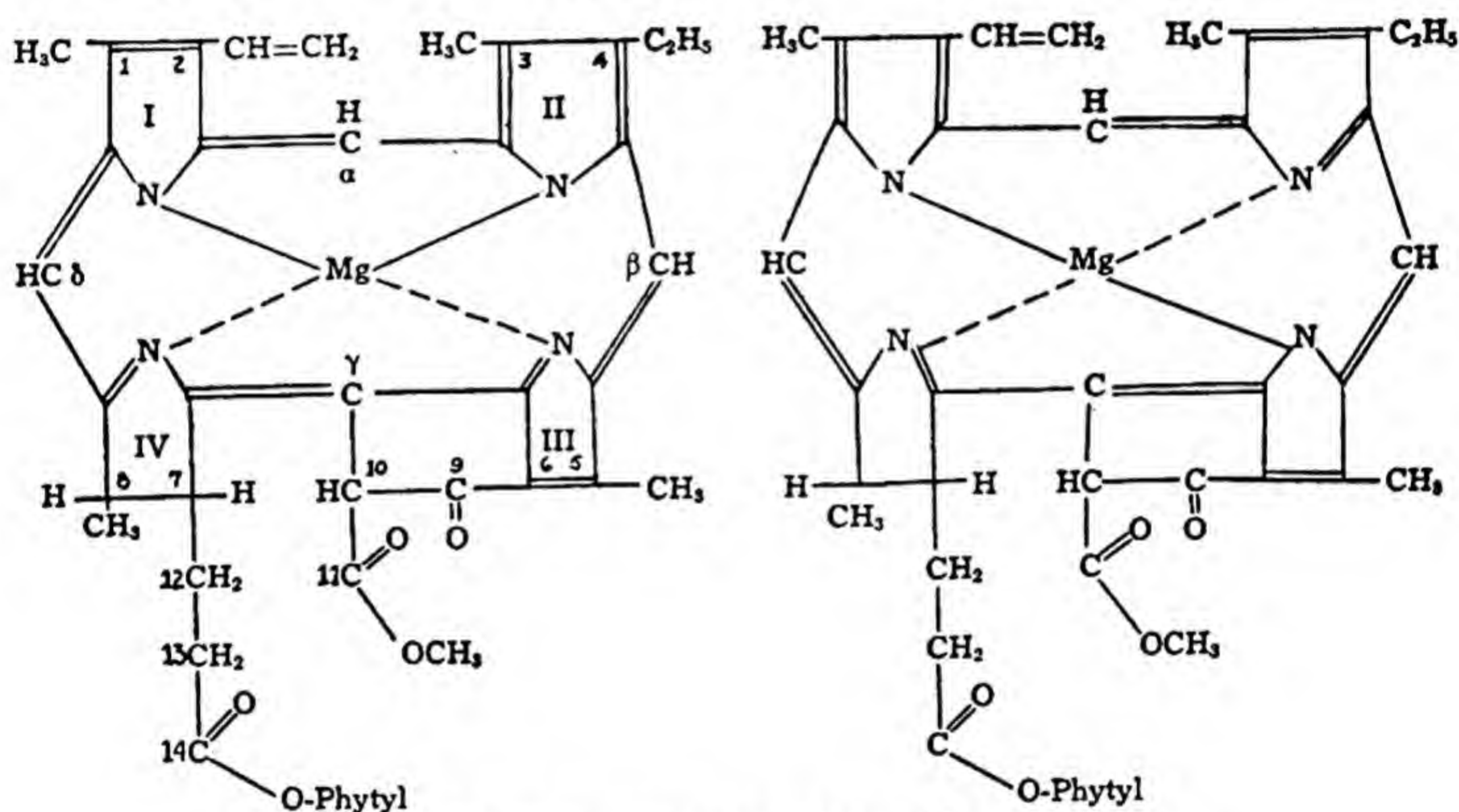
Spectral absorption curves:

In ether: (19, 52, 172).

In methanol: (85, 140). Figure 1.

In benzene: (34).

Molecular structure: The following structural formulas and system for numbering the carbon atoms are those proposed by Fischer (34). An alternative system for numbering the carbon atoms has been proposed by the International Union of Chemistry (111). Phytyl alcohol, which forms an ester group in the chlorophyll molecule, is optically active (72).



Alternative structures for chlorophyll *a*

Chlorophyll *a'*

Principal sources: Found in traces in some plants that contain chlorophyll *a*, probably as a result of isomerization during extraction of the pigments. Found in amounts equivalent to about 20 per cent of chlorophyll *a* in solutions that have been heated (139).

Formula: Probably $C_{55}H_{72}O_5N_4Mg$.

Optical rotation and number of double bonds not determined.

Absorption maxima ($m\mu$):

In methanol: —, 665.5 (88).

Chlorophyll *b*

Principal sources: Comprises about 20 to 40 per cent of the total

chlorophyll of green algae (Chlorophyta), euglenas (Euglenophyta), ferns, mosses, and higher plants.

Formula: $C_{55}H_{70}O_6N_4Mg$.

Optical rotation: $[\alpha]_{720\text{ m}\mu}^{25^\circ} = -267^\circ$ (130).

Number of double bonds: 16 including 2 ester groups.

Absorption maxima (m μ):

In ether: 455, 642.5 (19, 172).

In methanol: 470, 650 (85, 140). Figure 1.

Molecular structure: Identical with that of chlorophyll *a* except that an aldehyde ($-CH=O$) group occurs at position 3 in place of the methyl ($-CH_3$) group (34).

Chlorophyll b'

Principal sources: Found in traces in some plants that contain chlorophyll *b*, probably as a result of isomerization. Found in solutions of chlorophyll *b* after exposure to heat (139).

Formula: Probably $C_{55}H_{70}O_6N_4Mg$.

Absorption maxima (m μ):

In methanol: —, ca. 650.5 (88).

Chlorophyll c (Chlorofucine) (Chlorophyll γ)

Principal sources: Found in small amounts along with chlorophyll *a* in diatoms, dinoflagellates, and brown algae.

Formula and molecular structure unknown.

Absorption maxima (m μ):

In ether: 446, 579.5, 627.

In methanol: 450, 624.

Spectral absorption curve in methanol (141). Figure 1.

Chlorophyll d

Principal sources: Found in very small amounts along with chlorophyll *a* in most red algae but up to 25 per cent of the total chlorophyll in one species, *Rhodochorton rothii*.

Formula and molecular structure unknown; contains magnesium.

Absorption maxima (m μ):

In ether: 445, 686.

In methanol: 457, 696.

Spectral absorption curve in methanol (88). Figure 1.

Chlorophyll d'

Principal sources: Found in solutions of chlorophyll *d* as the result of isomerization.

Absorption maxima (m μ):

In methanol: —, 697 (88).

Isochlorophyll d

Principal sources: Found in solutions of chlorophyll *d* as the result of isomerization.

Absorption maxima (m μ):

In methanol: —, 661 (88).

Isochlorophyll d'

Principal sources: Found in solutions of chlorophyll *d* as the result of isomerization.

Absorption maxima (m μ):

In methanol: —, 661.5 (88).

Chlorophyll e

Principal sources: Found in small amounts along with chlorophyll *a* in a yellow-green alga (*Tribonema bombycinum*).

Absorption maxima (m μ):

In methanol: 415, 654 (138).

Unnamed Chlorophyll

Detected spectroscopically in extracts of a *Datura* mutant (61).

Protochlorophyll

Principal sources: Found in traces in etiolated seedlings, in etiolated sprouts, and in seeds of certain Cucurbitaceae (34, 42, 62, 110, 113).

Formula: $C_{55}H_{70}O_5N_4Mg$.

Number of double bonds: 16 including 2 ester groups.

Absorption maxima (m μ) (and absorption curve):

In ether: 571, 621 (113).

Molecular structure: Similar to that of chlorophyll *a* but dehydrogenated at carbon atoms 7 and 8 with slight shift in position of double bonds (34).

Bacteriochlorophyll

Principal sources: Found in purple sulfur bacteria. May exist in several forms, bacteriochlorophylls *a* and *b* (34, 116).

Formula: $C_{55}H_{74}O_6N_4Mg$.

Number of double bonds: 14 including two ester groups.

Absorption maxima (m μ) (and absorption curve):

In ethanol: 775 (76).

In methanol: 400, 605, 770 (43).

Molecular structure: Similar to that of chlorophyll *a* except that the vinyl group ($-\text{CH}=\text{CH}_2$) at position 21 is changed to an acyl group ($-\text{C}-\text{CH}_3$) and the double bond at carbon



atoms 3 and 4 is probably hydrogenated (34).

Bacterioviridin

Principal sources: Found in green sulfur bacteria.

Formula: Unknown.

Absorption maxima (m μ) (and absorption curve):

In ethanol: 670 (76).

Protoporphyrin 9

Principal source: Found in a chlorophyll-free *Chlorella* mutant.

Assumed to be a metabolic precursor of chlorophyll (47).

PROPERTIES COMMON TO XANTHOPHYLLS AND CAROTENES COLOR AND STRUCTURE

Both carotenes and xanthophylls are readily detected by their yellow or orange color and by their preferential solubility in fats and in fat solvents. Dilute solutions of these pigments are pale yellow, orange, or light red; concentrated solutions vary from light orange to deep red. Solutions of carotenoids exhibit from one to three absorption bands in the blue and green region of the spectrum (see Fig. 6.2). Crystals or solutions of the pigments react with concentrated sulfuric acid and with antimony trichloride in chloroform yielding blue products (89, 101, 131, 169, 170).

Molecules of most carotenoids contain forty atoms of carbon most of which are united in a long, multibranched chain. Composed of condensed isoprene units, this carbon chain contains a series of alternate single and double bonds. In many carotenoids, one or both ends of this carbon chain terminate in cyclohexenyl rings like those of ionone, the artificial odor of violets commonly formed when certain plants, *Trentepohlia*, or crystalline carotenes are permitted to oxidize in air. This occurrence of cyclic and of entirely aliphatic or acyclic structures complicates the nomenclature of the two types of pigments and necessitates use of an involved system for numbering the carbon atoms forming the skeleton of the molecule, as shown by formulas in the following section (Comm. Biochem. Nomen.). A "cipher" system, illustrated in connection with the formula for β -carotene, has also been proposed (26). This cipher system, like the usual nomenclature of organic compounds, is inapplicable to new substances whose structure is unknown or is incompletely established. These problems in nomenclature emphasize the great

differences that have developed between the objectives of organic chemists and those of physiologists and biochemists.

Color in the carotenoids is related to the outstanding structural feature of the molecules, the system of alternate single and double bonds, which usually contains from ten to fourteen unsaturated linkages as illustrated by formulas for individual carotenoids in the following section. The greater the number of these conjugated

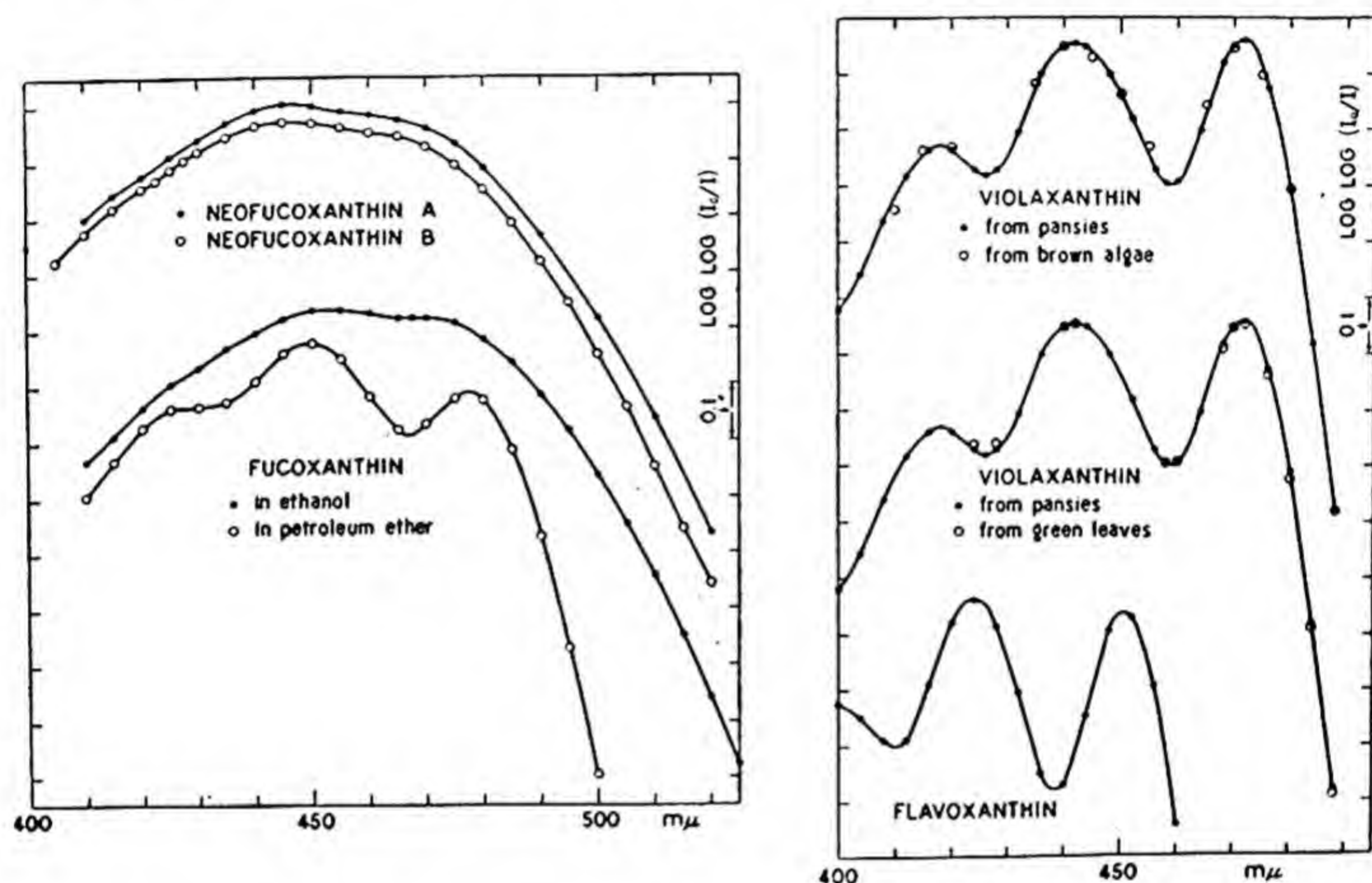


FIG. 6.2—Characteristic spectral absorption curves of several xanthophylls dissolved in 95 per cent ethanol and of fucoxanthin dissolved in petroleum ether (142).

double bonds, the redder and the more intense the color and the more pronounced the absorption bands.

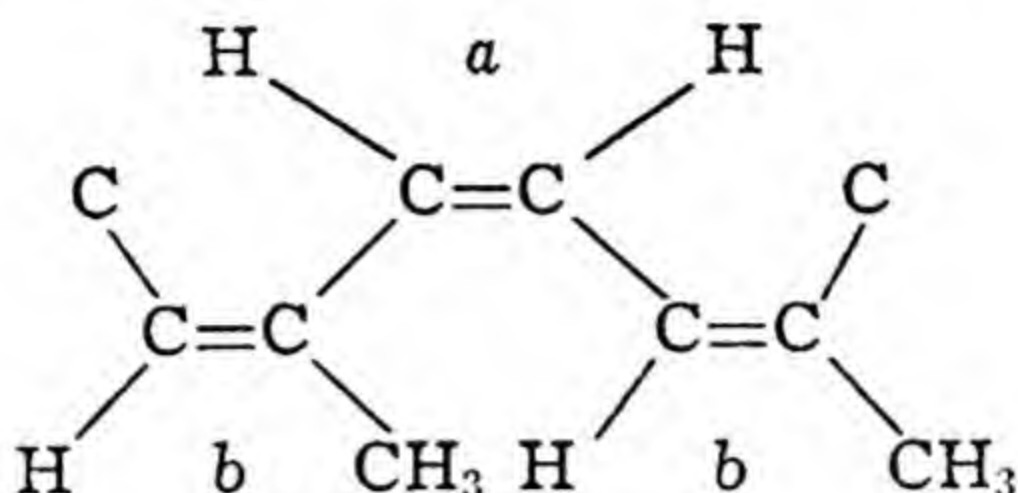
Many of the carotenoids differ from one another only in the number and arrangement of the double bonds. A few differ in the spatial arrangement of the groups adjacent to the double bonds. Others differ in the number of terminal rings and, as in the xanthophylls, in the number and function of the oxygen atoms.

The presence of unsaturated carbonyl groups ($>C=O$) in a carotenoid molecule usually reduces the sharpness of the absorption bands. Moreover, pigments containing these carbonyl groups exhibit less distinct absorption bands when dissolved in alcohols than when dissolved in petroleum ether. This effect of solvents on the shape of the absorption curves is illustrated by the absorption properties of fucoxanthin shown in Figure 6.2. Hydroxyl groups

(—OH) have little effect upon the spectral properties of the conjugated, polyene systems.

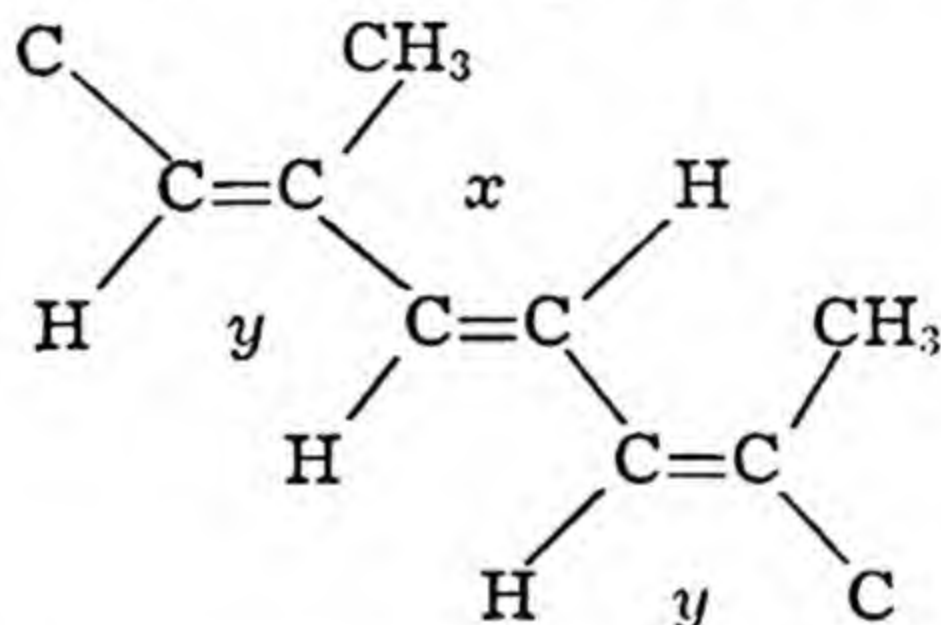
ISOMERIZATION

Groups at either side of each double bond can exist in two spatial arrangements, *cis* and *trans*, that are shown by the following partial formulas:



cis arrangements:

- a*—presumed to be relatively unstable at room temperature
- b*—relatively stable at room temperature



trans arrangements:

- x* and *y*—quite stable at room temperature.

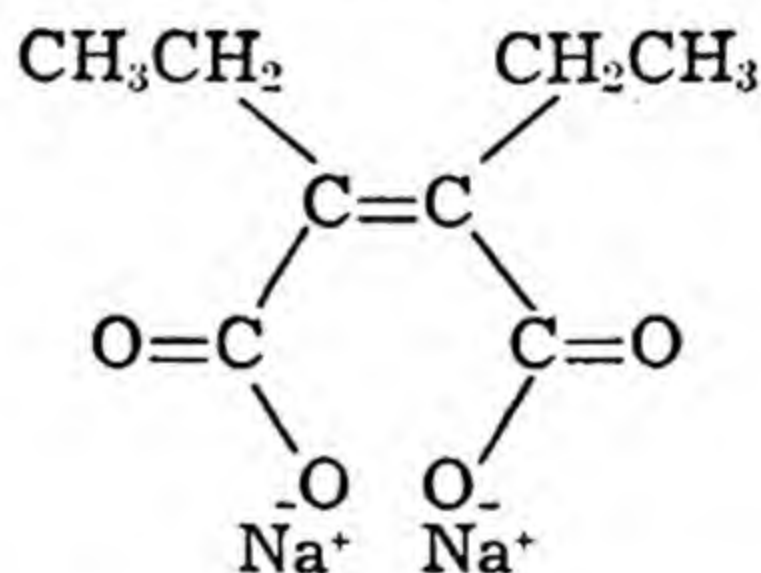
Spatial isomers that contain various arrangements of these *cis* and *trans* double bonds are separable from one another only by adsorption in Tswett columns. Many of them have been named by use of the prefix “neo—” with the name of the all-*trans* carotenoid from which they are derived (170).

Carotenoid pigments found in chlorophyllous tissues usually contain all double bonds in the *trans* arrangements. If solutions of these all-*trans* isomers are heated or treated with iodine in light, some of the isomers with one or more double bonds in *cis* arrangement are formed, although the relatively most stable, all-*trans* isomer usually predominates (170). This isomerization of the all-*trans* pigments is reflected in a slight shift of the spectral absorption maxima toward shorter wave lengths (131). Obviously each double bond in *cis* arrangement has less effect upon absorption of light by the isomer than the same double bond in *trans* configuration (170).

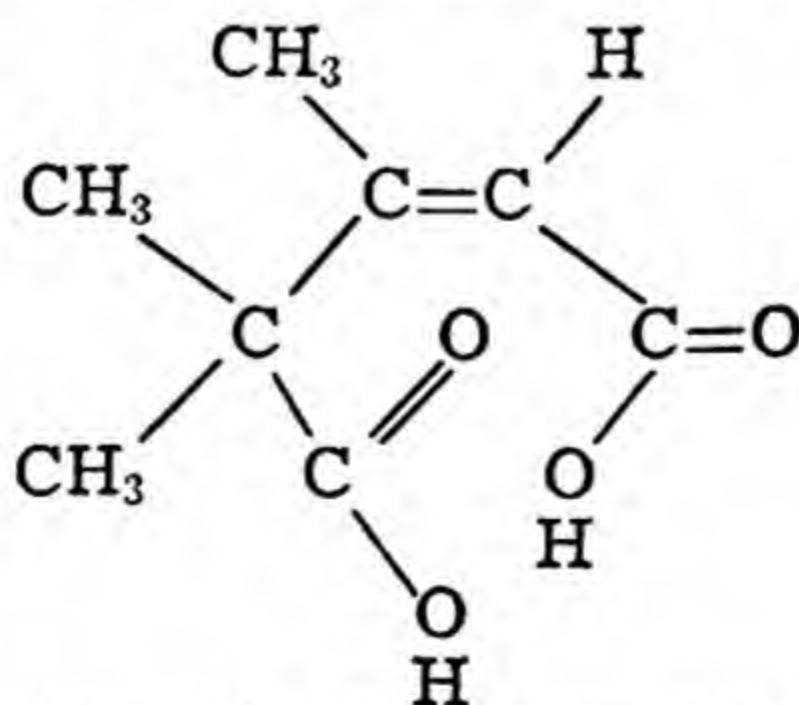
As each of the ten or more double bonds might occur in *cis*

and *trans* forms, the number of spatial isomers obtainable from each carotenoid pigment should be very great. In the isomerization mixtures, however, only a few isomers are found in significant quantities, an indication that some are relatively much more stable than others. This conclusion is supported further by isomerization of a few native pigments that contain many of the double bonds in *cis* arrangement. Treated with isomerization catalysts, solutions of these poly-*cis* carotenoids yield isomerization mixtures similar to those obtained from the all-*trans* pigments (170).

When structural groups adjacent to a double bond are crowded together as shown by bond *a* of the above *cis* partial formula, the molecule is supposed to be so unstable that these groups will rearrange spontaneously to the *trans* form (102). For this reason, formulas with these labile *cis* structures have not been considered for any of the natural carotenoid pigments. Yet, *a priori*, there is no reason to believe that pigments with these structures might not be synthesized and protected by the specialized tissues of living organisms. Whether or not double bonds with this labile *cis* configuration will rearrange spontaneously in solution to the *trans* form also appears to be an unsettled question. Analogous isomers with even larger groups that might interfere with one another do exist in *cis* as well as in *trans* arrangements; for example:



cis—Diethylmaleic acid sodium salt (9);



cis—Trimethylglutaconic acid (9).

As with the chlorophylls, particular geometrical isomers of the carotenoids are undoubtedly stabilized and protected within the

plant tissues. If this were not so, a mixture of isomers would always be obtained from plants instead of the one or two that usually abound, and the labile poly-*cis* isomers would not be found in fruits.

CHEMICAL REACTIONS

Carotenoid pigments undergo a variety of oxidation and reduction reactions that are characteristic of the polyene system. In solution these pigments may be hydrogenated to weakly colored or colorless substances, and they may be dehydrogenated to more deeply colored pigments. These reactions are not readily reversible, and they have not been observed in living cells.

In addition to the reactions of the polyene system common to all carotenoids, xanthophylls undergo chemical changes characteristic of the hydroxy, epoxy, and keto groups. These reactions (esterification and oxidation of hydroxyl groups, and reduction of keto groups) may sometimes be utilized to differentiate xanthophylls from carotenes.

DIFFERENTIATION BETWEEN CAROTENES AND XANTHOPHYLLS

Because carotenes, the polyene hydrocarbons, differ from xanthophylls, the oxygen derivatives of carotenes, with respect to chemical composition, purified pigments of each group can be distinguished by combustion analysis. If, however, only very small quantities of the pigments are available or if the pigments can not be purified, partition of the carotenoids between two immiscible solvents often serves to differentiate between the polyene hydrocarbons and their oxygen derivatives. Xanthophylls and their esters are extractable from petroleum ether with 85 per cent phosphoric acid; carotenes are not extractable. Most xanthophylls, including those produced by saponification of esters, are also extractable from petroleum ether with 90 to 95 per cent methanol, whereas carotenes are not extractable.

In Tswett columns of powdered sugar, most carotenes are less adsorbed than hydroxy carotenes and keto carotenes. This adsorption method, therefore, provides a rapid and sensitive means for differentiation between many carotenes and xanthophylls (135).

DESCRIPTION AND IDENTIFICATION OF THE INDIVIDUAL CAROTENOID PIGMENTS

Individual carotenes and xanthophylls have usually been detected and have often been isolated or purified by adsorption in Tswett columns. In view of the small quantities of material usually isolated by adsorption, descriptions of the purified carotenoids have frequently been based upon determination of relatively few properties selected in relation to the aims of the individual workers. Organic chemists have been interested principally in reactions that provide evidence of molecular structure (89). Investi-

gators in the field of photosynthesis have been concerned largely with spectral absorption properties, and for identification of the pigments they have relied upon comparative chromatographic adsorption methods, such as the position in adsorption columns relative to common pigments (135, 141, 142). Results obtained in one field are not readily applicable or acceptable in the other.

When encountered in a new source, a pigment that has been incompletely described is difficult to recognize. In many instances it can be identified with certainty only by reisolation from the original source. Occasionally, a single carotenoid isolated from unrelated sources has been given several names. Examples of such confusion, which are described in some detail in the section dealing with properties of the individual xanthophylls, are: peridinin, later called sulcatoxanthin; spirilloxanthin, later called rhodoviolascins; and violaxanthin, later called eloxanthin and xanthophyll-epoxide.

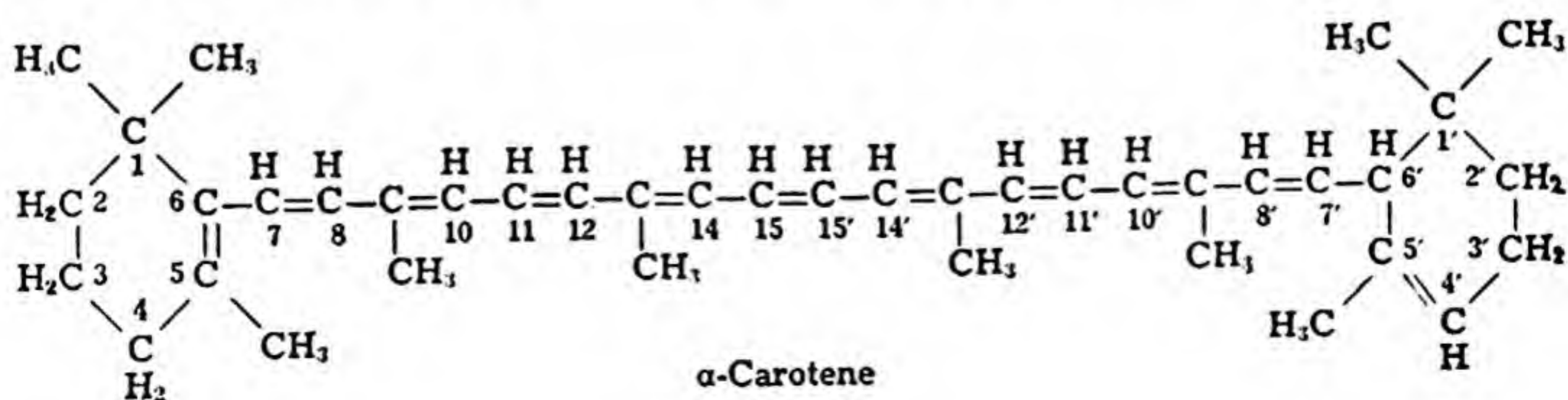
SOURCES AND PROPERTIES OF INDIVIDUAL CAROTENES

α -Carotene— $C_{40}H_{56}$. This carotenoid hydrocarbon occurs in small amounts in leaves of many plants and in the thalli of some algae. It is the major carotene of certain green algae of the order Siphonales but not of other green algae (138), and it is also the principal carotene of a few red algae.

α -Carotene is readily prepared from the carotene of carrots by adsorption in columns containing a mixture of magnesia and siliceous filter aid (135). Adsorption in columns also serves for the quantitative separation of α -carotene from other carotenoid hydrocarbons (3).

Spectral absorption curves, which form the basis of spectrophotometric methods for identification of α -carotene and for estimation of the quantity of pigment in solutions, have been determined in a variety of solvents: λ_{max} in ethanol, 446, 476 m μ (7, 74, 124, 154, 170, 175).

The molecule of α -carotene contains eleven double bonds, with ten of these in a conjugated system. There is but one asymmetric carbon atom, and the pigment is dextrorotatory (131). The molecular formula and a widely adopted numbering system proposed by Karrer are as follows (Comm. Biochem. Nomen.):

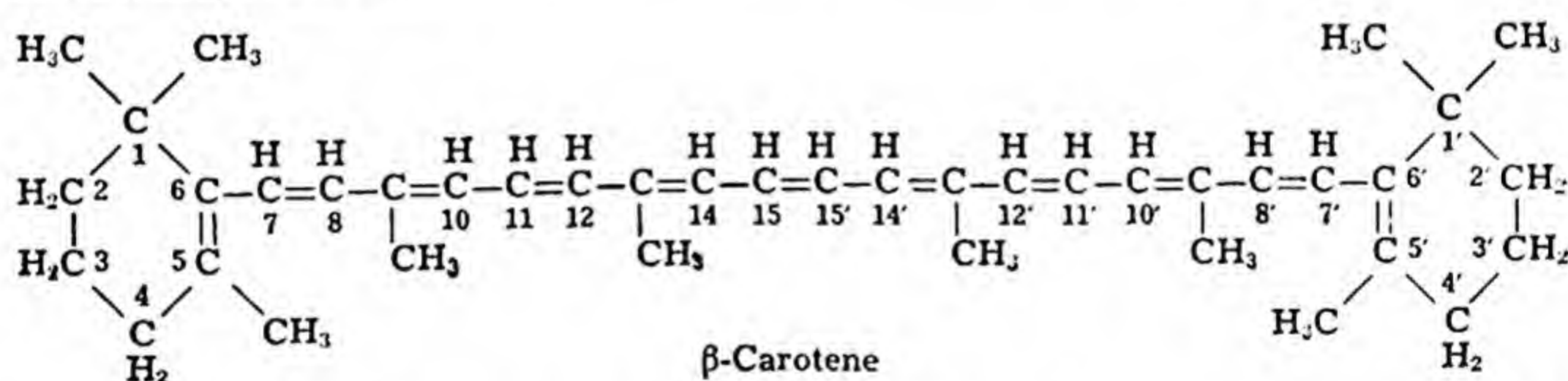


Through treatment of α -carotene with very strong alkalis, such as sodium alcoholates, the isolated double bond has been shifted to form small quantities of the isomeric β -carotene with all double bonds in conjugation. The reverse reaction, conversion of β -carotene to α -carotene, was not observed (66). In living plants, the ratio of α -carotene to β -carotene appears to be controlled by genetic processes that are unrelated to this isomerization caused by alkali.

β -Carotene— $C_{40}H_{56}$. Except for a few red algae and some green algae of the order Siphonales, β -carotene is the principal carotenoid hydrocarbon of all organisms found to contain chlorophyll *a* (137, 138). This carotene is readily prepared from fresh leaf material or from carrot root carotene by adsorption in columns of lime (170) or of magnesia (135). Methods for the quantitative determination of carotene in plant material based upon partition of the pigments between immiscible solvents and upon adsorption in Tswett columns have been summarized by Wilkes (166) and by the Association of Vitamin Chemists (3).

Spectral absorption curves of β -carotene resemble those of α -carotene; but the absorption maxima are not so well defined, and they are shifted to longer wave lengths; λ_{\max} in ethanol, 452, 482 m μ (3, 74, 124, 131, 133, 169, 170, 175).

With respect to chemical structure, β -carotene is very similar to α -carotene. Its eleven double bonds occur in conjugation. The molecule is symmetrical and is optically inactive as indicated by the following formula:



The "cipher" proposed for β -carotene by Dyson is: C18.1, 18[2AC6.1, 1, 3 C.2E].3, 7, 12, 16C.1(2)17E. Here the numbers refer to the carbon atoms in the longest chain, not to the numbers shown in the structural formula.

γ -Carotene— $C_{40}H_{56}$. Traces of γ -carotene occur in many green plants. The amounts of this pigment increase significantly in gametes of some green algae and in the reproductive organs of certain stone-works (65, 138). γ -Carotene is a minor constituent of carrot carotene from which it can be prepared by chromatographic adsorption (135). Spectral absorption maxima occur at longer wave lengths than the maxima of β -carotene; λ_{\max} in ethanol, 462, 494 m μ .

The molecule of γ -carotene contains twelve double bonds, eleven

in a conjugated system. There is one cyclohexenyl ring like those of β -carotene; the other end of the molecule is aliphatic with a terminal isopropylidene group.

ϵ -Carotene. Small amounts of this polyene pigment have been isolated from one species of diatom, *Navicula torquatum*, and from a green alga, *Bryopsis corticulans*. The chemical composition and molecular structure are not known. Absorption maxima occur at short wave lengths; λ_{max} . in ethanol, 440, 470 m μ (138).

Flavacin. Very small amounts of flavacin accompany β -carotene in blue-green algae; λ_{max} . in petroleum ether, 428, 459 m μ (148).

Rhodopurpurin. Crystals of rhodopurpurin have been obtained from *Rhodovibrio* bacteria; λ_{max} . in petroleum ether, 472, 502 m μ (73).

Flavorhodin. This carotenoid hydrocarbon was isolated in small amounts from *Rhodovibrio* bacteria; λ_{max} . in ethanol, 443, 472 m μ (73, 75).

SOURCES AND PROPERTIES OF INDIVIDUAL XANTHOPHYLLS

Cryptoxanthin— $\text{C}_{40}\text{H}_{55}\text{OH}$ —3-Hydroxy- β -carotene. Traces of this xanthophyll occur in leaves and in many green algae. Pure cryptoxanthin is most readily obtained from the red calyx of the Chinese lantern, *Physalis alkekengi*, by saponification of the native ester. Spectral absorption properties of the cryptoxanthin are almost identical with those of the parent β -carotene; λ_{max} . in ethanol, 452, 482 m μ (79, 89, 131, 175).

Zeaxanthin — $\text{C}_{40}\text{H}_{54}(\text{OH})_2$ — 3,3'-Dihydroxy- β -carotene. Very small quantities of zeaxanthin accompany lutein, the principal xanthophyll, in leaves and in green algae. Zeaxanthin is the principal xanthophyll of a few red algae and of certain blue-green algae. This pigment forms a conspicuous, strongly adsorbed, red-orange band in columns of magnesia when petroleum ether plus 20–25 per cent acetone is used as solvent. Discovered in yellow corn, *Zea mays*, zeaxanthin is most easily prepared from the ester which occurs in relatively large amounts in the calyx of *Physalis*. Spectral absorption properties of zeaxanthin are similar to those of β -carotene and of cryptoxanthin; λ_{max} . in ethanol, 453, 480 m μ . Solutions of zeaxanthin are levorotatory (89, 131, 169, 170, 175).

Lutein— $\text{C}_{40}\text{H}_{54}(\text{OH})_2$ —3,3' Dihydroxy- α -carotene. Lutein, also called xanthophyll, is the major carotenoid pigment of higher plants, of ferns, mosses, stoneworts, and of most green algae and most red algae. It is readily prepared from leaves by a combination of partition and chromatographic adsorption methods. Spectral absorption

properties of lutein resemble those of the parent α -carotene; λ_{\max} . in ethanol, 447, 476 m μ (75, 89, 131, 169, 170, 175). Strong alkalies convert lutein into zeaxanthin in small yield (66).

Violaxanthin or *eloxanthin* or *xanthophyll-epoxide*?— $C_{40}H_{56}O_4$. This pigment of yellow pansies, *Viola tricolor*, occurs with lutein in leaves and in green algae. Preparations of the pigment from pansies and from leaves are chromatographically identical. Violaxanthin is readily obtained from leaves by adsorption of the extracts in columns of magnesia using petroleum ether plus about 25 per cent acetone as solvent. Under these conditions it is absorbed below the principal band of lutein (131, 142).

Violaxanthin gives an intense blue color when an ether solution is shaken with concentrated hydrochloric acid. Other pigments with almost identical spectral absorption properties (taraxanthin from dandelions, and dinoxanthin from dinoflagellates) do not exhibit this color reaction. The chemical relationship among these similar pigments is not known.

Spectral absorption maxima of violaxanthin, as shown by Figure 6.2, occur at short wave lengths; λ_{\max} . in ethanol, 442, 472 m μ (75, 131,

143). Recent evidence indicates that epoxy groups, $\begin{array}{c} \text{O} \\ \diagup \quad \diagdown \\ \text{C} \text{---} \text{C} \end{array}$, may be present in the violaxanthin molecule (64, 69).

Recently it has been claimed that violaxanthin from pansies and violaxanthin from leaves yield different pigments when treated with acids; hence, the leaf violaxanthin has been called xanthophyll-epoxide (68). But this result could not be confirmed. Exposed to acid, violaxanthin from the two sources has been found to yield unstable flavoxanthins which are quickly transformed into auroxanthins. On the basis of all these results, violaxanthin should be the preferred name for this labile xanthophyll of leaves (see flavoxanthin).

Eloxanthin. Reported as a major xanthophyll of *Elodea canadensis* by Hey, eloxanthin has since been found to be a minor constituent accompanying lutein and β -carotene. This strongly adsorbed pigment with absorption maxima at 444, 472, and 502 m μ in carbon disulfide is apparently identical with violaxanthin (71, 138).

Flavoxanthin— $C_{40}H_{56}O_3$. Traces of flavoxanthins occur in leaves of higher plants. This xanthophyll is most readily prepared from buttercups in which it was first discovered. Its spectral absorption maxima, which are exceptionally well defined as illustrated by Figure 6.2, occur at short wave lengths; λ_{\max} . in ethanol, 422, 452 m μ (64, 69, 77, 131).

Recent evidence indicates that flavoxanthin contains a furanoid

ring adjacent to the cyclohexenyl ring so common in carotenoids (64, 69). Although leaf flavoxanthins yield a blue color with hydrochloric acid, some flavoxanthin-like pigments from flowers do not yield a blue color with this acid (142). Obviously these pigments from the two sources are different. It is possible that they may differ in chemical composition, because the flavoxanthin of flowers contains but three atoms of oxygen whereas the flavoxanthin-like pigments of leaves are probably derived from violaxanthin which contains four atoms of oxygen (Strain, unpubl.).

Neoxanthin— $C_{10}H_{56}O_4$. This xanthophyll occurs in its most stable, spatial form in green algae and in higher plants. Usually isolated by adsorption of extracts of leaves in columns of powdered sugar or by adsorption of saponified leaf extracts in columns of heat-treated siliceous earth, Celite, neoxanthin is one of the most adsorbed xanthophylls found in the green parts of plants. Solutions of purified preparations of neoxanthin in ether do not form a blue product when treated with concentrated hydrochloric acid. Spectral absorption curves show pronounced absorption maxima at wave lengths intermediate between those of the absorption maxima of lutein and of violaxanthin; λ_{max} in ethanol, 437, 467 m μ (131, 138, 142).

Fucoxanthin— $C_{40}H_{54-60}O_6$. Observed first in brown algae, *Fucus* of the Phaeophyceae, fucoxanthin has since been found to be the principal xanthophyll of diatoms, Bacillariophyceae. This xanthophyll is readily obtainable from brown algae by partition of the pigments between immiscible solvents or by adsorption of the pigments in columns of powdered sugar. Fucoxanthin is decomposed and decolorized rapidly by alcoholic potassium hydroxide, and it is altered slowly by adsorption on alkaline adsorbents such as lime or magnesia (55, 89, 101, 142, 169).

As shown already by Figure 6.2, spectral absorption curves of fucoxanthin dissolved in polar solvents are poorly defined relative to the curves for the pigment dissolved in nonpolar solvents, an indication of the presence of one or more ketonic groups in the molecule; λ_{max} in ethanol, ca. 452, 470 m μ ; in petroleum ether, 449, 477 m μ (24, 75, 142, 154).

In solution, fucoxanthin undergoes reversible isomerization yielding two isomers, neofucoxanthin A and B, which are more adsorbed than the fucoxanthin itself. Even if diatoms and brown algae are extracted quickly at low temperature, appreciable quantities of these isomers are present in the extracts; hence, they may be normal constituents of the living organisms (142).

A structural formula depicting fucoxanthin as a hydroxyl derivative of capsorubin (55) is open to question. In petroleum

ether, absorption maxima of capsorubin occur at 474 and 507 m μ . Introduction of hydroxyl groups into the capsorubin molecule would not be expected to shift these absorption maxima to correspond with those of fucoxanthin; namely, to 449, 477 m μ .

Diatoxanthin. This xanthophyll with spectral absorption and chromatographic adsorption properties similar to those of zeaxanthin has been isolated in small quantities from diatoms, the only known source; λ_{max} in ethanol, 453, 481 m μ (142).

Diadinoxanthin. Both diatoms and dinoflagellates yield this xanthophyll which exhibits spectral absorption curves remarkably like those of lutein; λ_{max} in ethanol, 448, 478 m μ . Mixtures of this pigment and lutein are readily separable in adsorption columns of powdered sugar, the diadinoxanthin forming the upper band (142).

Dinoxanthin. Dinoflagellates yield a small quantity of dinoxanthin which exhibits spectral absorption properties similar to those of violaxanthin and taraxanthin; λ_{max} in ethanol, 441.5, 471 m μ . In columns of powdered sugar and with petroleum ether plus a little propanol as solvent, dinoxanthin is adsorbed below violaxanthin and above taraxanthin (142).

Peridinin or *sulcatoxanthin*— $\text{C}_{40}\text{H}_{52}\text{O}_8$. Isolated first from the dinoflagellate, *Peridinium*, later from the sea anemone, *Anemonia sulcata*, peridinin is most readily prepared from the symbiotic alga of the sea anemone, *Bunodactis xanthogrammica*, which is available the year around. This xanthophyll exhibits spectral absorption properties characteristic of ketonic pigments; λ_{max} in ethanol, ca. 475 m μ ; in petroleum ether, 452, 486 m μ . In solution in *n*-propanol at 100°C., it undergoes reversible isomerization, yielding small quantities of a similar, more adsorbed isomer, neoperidinin.

Peridinin resembles fucoxanthin with respect to its spectral absorption properties, but it is readily separated from fucoxanthin by adsorption of a mixture of the two pigments in columns of powdered sugar wherein it forms the upper band. Unlike fucoxanthin, peridinin does not yield a clear blue color when the ether solution is shaken with concentrated hydrochloric acid (142).

Myxoxanthin (also called *aphanin* and *calorhodin* α)— $\text{C}_{40}\text{H}_{54}\text{O}$. All blue-green algae examined thus far yield considerable proportions of this weakly adsorbed, ketonic xanthophyll which has also been reported in small amounts in the red form of a green alga, *Hämatococcus pluvialis*; λ_{max} in carbon disulfide, 489 m μ (138, 149). A structural formula recently proposed indicates that this xanthophyll is a 4-keto-2,3-dehydro- β -carotene (70, 89).

Myxoxanthophyll (known also as *aphanizophyll* and *myxorhodin*). This abundant, exceptionally strongly adsorbed, ketonic xanthophyll has been found in all blue-green algae (70, 138, 149) and in *Hämatococcus* (149).

Siphonaxanthin. Green algae of the order Siphonales yield significant quantities of this ketonic pigment that closely resembles fucoxanthin with respect to spectral absorption properties; λ_{\max} in ethanol, ca. 455 m μ ; in petroleum ether, 452, 480 m μ . In columns of powdered sugar and with petroleum ether plus about 1 per cent propanol as solvent, siphonaxanthin is absorbed far above fucoxanthin and peridinin. Mixtures of these three similar pigments are, therefore, readily separable in the adsorption columns. Unlike fucoxanthin, siphonaxanthin is resistant to alkalies, and it does not yield a blue color when shaken with ether and concentrated hydrochloric acid (138).

Siphonein. In most algae containing siphonaxanthin one finds about equivalent amounts of a spectroscopically similar carotenoid which is converted into siphonaxanthin by saponification with alcoholic potassium hydroxide. This conversion by alkali suggests that this pigment, siphonein, is an ester or partial ester of siphonaxanthin. Siphonein is separated with difficulty from fucoxanthin by adsorption in columns of powdered sugar and could easily be confused with this principal xanthophyll of diatoms and brown algae (138).

4 *Rhodoxanthin*—3,3'-Diketo-4,5-4',5'-dehydro- β -carotene. Bronze colored, winter needles of the ornamental conifer *Cryptomeria japonica* are a convenient source of this ketonic xanthophyll. In ethanol, rhodoxanthin exhibits a single, indistinct, spectral absorption maximum; in petroleum ether there are two maxima at 489 and 524 m μ (75, 78, 82, 89).

Euglenarhodon— $C_{40}H_{48}O_4$. This ketonic xanthophyll, isolated in esterified form from a red Euglena, *Euglena heliorubescens*, and from a red *Hämatococcus*, resembles or is identical with astacene, a ketonic carotenoid isolated from crustaceans (146, 149). A similar ketonic pigment isolated from the red form of *Protosiphon* also resembles astacene but is chromatographically distinct from the pigments extracted from crab shells. The chemical relationship among these similar ketonic pigments is obscure (138).

Spirilloxanthin or *rhodoviolascin* or *bacterio-erythrin* or *bacteriopurpurin*— $C_{40}H_{54}(OCH_3)_2$. This unusual dimethoxy carotenoid is the principal pigment of purple bacteria, *Rhodospirillum rubrum*, and of certain *Rhodovibrio* bacteria. The pigment can be purified

by chromatographic adsorption, but it is extremely labile and undergoes rapid, spontaneous, reversible isomerization at room temperature. Absorption maxima of spirilloxanthin occur nearer the red region of the spectrum than those of other carotenoid pigments; λ_{max} in petroleum ether, 461, 493, 528 m μ . This longer wave length of the spectral absorption maxima can be attributed to the presence of thirteen conjugated double bonds in the pigment molecule (67, 73, 75, 103, 151, 152).

Rhodopin— $\text{C}_{40}\text{H}_{56-58}\text{O}$. Isolated from *Rhodovibrio* bacteria by chromatographic adsorption, rhodopin exhibits spectral absorption maxima at shorter wave lengths than does spirilloxanthin; λ_{max} in ethanol, 445, 474, 505 m μ (73, 75). This pigment, which is the predominant xanthophyll of certain *Athiorhodaceae*, may be a monomethoxy carotene (151).

PROPERTIES CHARACTERISTIC OF PHYCOBILINS COLOR AND MOLECULAR STRUCTURE

Spectral absorption properties of the phycobilins are due to a metal-free, relatively small, linear, tetrapyrrolic, chromophoric group in combination with a globulin-like protein. Hydrolysis of these phycochromoproteins with strong acids results in simultaneous oxidation of the colored bilins to colorless products unless the reaction mixture is protected from oxygen (12).

The molecular weight of the natural phycocyanins and phycoerythrins ranges from 263,000 to 291,000. It is decreased a great deal by weak acids and by very weak alkalies (31, 143). Alteration of the molecular weight by small changes in hydrogen ion concentration apparently involves reactions of the colorless protein, because these variations of the molecular weight are not associated with significant changes in the color of the pigments.

Most of the phycobilins are strongly fluorescent, emitting orange or red light (22). Fluorescence of the phycobilins from red algae is decreased rapidly by strong oxidizing agents, and it is decreased slowly by exposure of the solutions to air. Fluorescence of the phycobilins from blue-green algae is but slightly affected by oxidizing agents (138).

DIFFERENTIATION BETWEEN PHYCOERYTHRINS AND PHYCOCYANINS

Differences between phycoerythrins and phycocyanins are not very clear cut. Most phycoerythrins are red; phycocyanins are blue. As a rule, phycocyanins are more soluble than phycoerythrins. Pigments of the two groups are separable by crystallization from ammonium sulfate solutions. They are also separable by adsorption in strips of filter paper (81) and in columns of starch (136).

In red algae, phycoerythrins occur with the fat-soluble pigments within the microscopic chloroplasts, but in blue-green algae the phycocyanins occur in more diffuse, submicroscopic structures along with the chlorophylls and carotenoids. The condition of the phycoerythrins in the chloroplasts, their chemical relationship to phycocyanins, and the chemical relationship among phycoerythrins from different sources are not known with certainty (12).

SOURCES AND PROPERTIES OF INDIVIDUAL PHYCOBILINS

INDETERMINATE PROPERTIES

There are but two principal, well-defined, proteinaceous pigments of the red and the blue-green algae. These pigments are: *r*-phycoerythrin, $\lambda_{\text{max.}}$ 497.5, 540, 566 m μ , of the Rhodophyceae (143) and *c*-phycocyanin, $\lambda_{\text{max.}}$ 615 m μ , of the Cyanophyceae (144). A few of the red algae yield principal phycoerythrins that exhibit indistinct spectral absorption bands and that differ in their fluorescence capacity. Because similar, weakly fluorescent pigments are formed from the most abundant phycoerythrin by oxidation, the natural, nonfluorescent phycoerythrins may represent alteration products of the strongly fluorescent *r*-phycoerythrin. Even though the spectral differences among the extracted phycoerythrins are very striking (143), all these pigments can be arranged in a series showing a gradual decrease in absorption capacity and in fluorescence intensity. Until more information is available, one can not be certain about the nature, the number, or the origin of these phycoerythrins that are believed to occur naturally. Progress in this aspect of the subject is hampered by the lack of analytical methods for the resolution of mixtures of closely related proteinaceous pigments.

There are two minor, fairly well-defined phycobilins. These are: *r*-phycocyanin, $\lambda_{\text{max.}}$ 553, 615 m μ , of many Rhodophyceae inhabiting the intertidal zone (145) and *c*-phycoerythrin, $\lambda_{\text{max.}}$ 552 m μ , of certain olive-green Cyanophyceae (12). Isolation of phycocyanins with two spectral absorption bands in the red region of the spectrum has been attributed, in part, to the presence of phycoerythrin in the phycocyanin, and in part, to alteration of the phycocyanin during the extraction and purification (13).

With respect to the number of spectral absorption bands, the *r*-phycoerythrin and the *r*-phycocyanin seem to be closely related, each pigment having two absorption maxima in the orange and red region of the spectrum. Similarly, the *c*-phycoerythrin and the *c*-phycocyanin have single absorption bands in the red region of the spectrum. In this respect, the two pigments from each source appear to be more closely related than is indicated by their present classification as different kinds of phycobilins.

FLORIDORUBIN

A single Rhodophycean species *Rytidhlaea tinctoria* contains the water-soluble, red floridorubin in its plastids in place of phycoerythrin. This floridorubin is not a protein. It contains about 9.5 per cent nitrogen and about 50 per cent oxygen. Solutions of the pigment in water exhibit a green fluorescence, and solutions in ether show spectral absorption bands at 454 and 480 m μ . The molecular structure of the pigment and its physiological role in the plastids have not been determined (33).

PROPERTIES OF THE PIGMENTS WITHIN THE CHLOROPLASTS

ABSORPTION SPECTRA OF LIVING PLANTS

The color of autotrophic plants depends upon the nature, the proportions, and the concentration of the pigments. It varies with the condition or state of the pigments in the chloroplasts and with the species and the environment.

Absorption of light by living plant material parallels the spectral absorption properties of solutions of the extracted pigments. In the green region of the spectrum where absorption by chlorophyll solutions is only about 3 to 5 per cent of the maximum absorption in the red region of the spectrum, light absorption by the living plant material may be as great as 20 per cent or more of the absorption in the red region of the spectrum (1, 16, 28, 29, 106, 160). This effect may result from the high concentration of the pigments in the chloroplasts which causes virtually complete absorption of the incident blue and red light and great absorption of the green light (138). It may also indicate that the chlorophylls and carotenoids occur in a special state or are segregated within the chloroplasts. It is of significance in connection with determination of the photosynthetic activity of the individual colored constituents of the chloroplasts, because distribution of the absorbed energy between the complementary pigments and the chlorophyll depends upon their relative absorption capacities in the plastids.

Chlorophyll *a* in the living cells of various plants exhibits a spectral absorption maximum at about 681 m μ ; that is, at slightly longer wave lengths than chlorophyll *a* in colloidal solution, in very concentrated solution, in solid form, or in leaves killed by freezing and thawing. This meager evidence indicates that the chlorophyll *a* may always occur in plants in the same labile, physical state which is probably different from that of chlorophyll in any artificial preparation. The difference between the wave lengths of the absorption maxima of bacteriochlorophyll in alcohol and in the living bacteria is even greater than the corresponding difference for chlorophyll *a* (43, 76).

STRUCTURE OF THE CHLOROPLASTS

An increasing amount of microscopical and ultramicroscopical evidence shows that chloroplasts have a highly specialized structure (48, 107, 109). The bright red color of many Rhodophyceae and the labile, brown color of the plastids of many Phaeophyceae, Bacillariophyceae, and Dinophyceae indicate that the pigments may occur within this structure in special states or in unique orientation. For example, the chlorophylls and carotenoids might occupy separate portions of the chloroplasts. Although knowledge of such a preferential distribution or orientation of the pigments in the plastids is of importance for calculation of the relative amount of the incident energy absorbed by each pigment, quantitative information regarding the orientation of the pigments is very scant.

Carotenoids and chlorophylls always occur in intimate association so that they may be parts of the same physiologically active structure. Phycobilins, particularly phycocyanins, must be weakly bound to the carotenoids and chlorophylls, because the water-soluble phycocyanin separates quickly from the green and yellow pigments when the plant cells are ruptured at 0° (136).

FLUORESCENCE OF CELLS AND OF CHLOROPLASTS

~^{not read} Compared to the intense fluorescence of chlorophyll in leaf extracts, the fluorescence of chlorophyll in living cells and in isolated chloroplasts is exceptionally weak, being only 0.005 to 0.15 per cent of the incident energy. In a given organism, fluorescence is proportional to light intensity, except during the first minutes after initial illumination. Of special interest is the observation that cells in vigorous, young cultures show little or no variation of the fluorescence in the first few minutes of illumination (117). Poisonous substances that supposedly affect the "dark" reactions of photosynthesis have little effect upon the fluorescence; substances that are presumed to retard the photochemical reactions tend to increase the fluorescence (41, 99, 158, 162). The weak fluorescence of chlorophyll in concentrated solutions and in the plastids may be due to absorption of the fluorescent light by the chlorophyll itself (174).

In diatoms, light of wave lengths absorbed principally by the carotenoid pigments stimulates the fluorescence of chlorophyll to the same extent as light of wave lengths absorbed by chlorophyll alone (25, 160). This effect may be attributed to transfer of absorbed energy from carotenoids to chlorophyll *a* (25), or, in part, to geometric orientation or segregation of the pigments (138). Chlorophyll *b* and chlorophyll *c* do not exhibit definite fluorescence bands within living organisms (22), an effect that may be due to the

segregation, to the physical state, or to the relative concentration of these pigments within the chloroplasts.

Compared to the intense fluorescence of their dilute aqueous solutions, phycobilins in living red algae and in living blue-green algae are weakly fluorescent. This weak fluorescence of the phycobilins, like that of the chlorophylls, may facilitate conservation of the energy absorbed by the pigments within the living cells.

From the results obtained thus far, much more work will be required in order to interpret all the fluorescence phenomena of autotrophic organisms (159). The results of Shiau and Franck show that special attention should be given to the age and condition of the photosynthesizing cells. The relationship between the fluorescence and the photosynthetic activity of living cells is worthy of further study, but control experiments on the fluorescence of pigments in very concentrated solution are also needed.

REACTIONS OF THE PIGMENTS AND PIGMENT FORMATION

(Few specific chemical reactions of the pigments have been discovered in living organisms. Although the chloroplast pigments are formed from colorless substances and, in many plants, are reconverted to colorless substances, the intermediate steps have yet to be elucidated. So far as is known, these reactions of the pigments usually occur slowly, relative to the production of organic matter by photosynthesis.

Formation of chloroplast pigments is influenced by many different conditions. Some plants form both carotenoids and chlorophylls in the dark; others form only carotenoids and traces of a green pigment called protochlorophyll. Deficiencies of mineral nutrients permit plastid development but inhibit chlorophyll formation either in light or in darkness.) Treatment of germinating seeds with streptomycin, an antibacterial, inhibits plastid development and thus retards formation of carotenoids and chlorophylls (15). Exposure of etiolated seedlings to warm water for a few minutes retards chlorophyll formation long after the plants have been brought to room temperature (131).

As etiolated seedlings contain carotenoids and traces of protochlorophyll and as they form chlorophyll quickly upon exposure to light, much attention has been given to the conditions that affect chlorophyll formation in them (62). In certain etiolated seedlings, chlorophyll synthesis has been resolved into at least two steps: a series of nonphotochemical or dark reactions which depend upon the normal oxidative metabolism of the cell and which lead to the formation of protochlorophyll, and a series of photochemical reactions which cause the disappearance of protochlorophyll with the

simultaneous appearance of chlorophyll. The nonphotochemical reactions are inhibited by circumstances that retard respiration: low temperatures (83), enzyme poisons as cyanide (168), and antioxidants as hydroquinone (134). The photochemical reactions are not inhibited by cyanide or by low temperatures (83, 168).

An action spectrum for the formation of chlorophyll in etiolated oat seedlings resembles the absorption spectrum of protochlorophyll in the red region of the spectrum. A pronounced maximum in the blue region of the action spectrum indicates that protochlorophyll also has an absorption band in this spectral region (42). In these experiments with oat seedlings, chlorophyll increased continually, so that finally it absorbed much more light than the protochlorophyll. From this fact and from the shape of the action spectrum one may postulate that light absorbed by the newly formed chlorophyll may also be effective in stimulating the synthesis of additional chlorophyll. For example, the effectiveness of red light in the formation of chlorophyll is nearly as great in the red spectral region of the chlorophyll *a* absorption maximum as in the orange region of the protochlorophyll absorption maximum.

Variations in the amounts of the carotenoid pigments in etiolated seedlings are without effect upon the action spectrum for chlorophyll formation. This result may be attributed to preferential exposure of the protochlorophyll to the incident radiation (42) or perhaps simply to a segregation of the pigments in the plastids. But neither of these conclusions can be reconciled with the action spectrum for inhibition of internode elongation in etiolated seedlings. This inhibition action spectrum, which has also been attributed to absorption of red light by protochlorophyll, shows no maximum in the blue region of the spectrum (46, 165).

✓ Reactions that lead to the formation of protochlorophyll and to the incorporation of magnesium into the chlorophyll molecule have yet to be discovered. The available evidence indicates that the intermediate products do not accumulate in large amounts (6, 125).

✓ In unrelated plants, chlorophylls may be formed by different reaction mechanisms. The first chlorophyll formed in etiolated seedlings exposed to light is chlorophyll *a* (45, 59, 87), the normal proportion of chlorophyll *b* appearing subsequently. In contrast to etiolated seedlings, green algae form green pigments in the dark and yield both chlorophyll *a* and chlorophyll *b* (96). Green algae that are deficient in chlorophylls, because of a shortage of sulfur compounds in the medium, form additional quantities of the green pigments in the dark when supplied with sulfur compounds (86). Inhibition of chlorophyll formation by various adverse environmental conditions and by many genetic factors provides further

indication of an involved mechanism for pigment synthesis. As exposure of seedlings to white light stimulates their respiration (165), the secondary effects of irradiation may also stimulate pigment synthesis.

The capacity of leaves to form chlorophyll depends upon their previous treatment. If a portion of an etiolated barley seedling is exposed to light for a day or so, the exposed portion becomes green; the unexposed portion remains yellow. If this partially green seedling is now returned to the dark for several days, the green portion fades very much. Upon exposure of the entire seedling to light, chlorophyll forms faster in the previously unexposed portion than in the exposed and faded portion.

Leaves vary greatly in the rate at which they lose chlorophyll in the dark. As a rule, young leaves bleach faster than older ones. The leaves of some species, as barley, may lose most of their green pigments after a few days in the dark; leaves of other species, as *Bryophyllum* and *Cycas*, may retain much pigment after several months in the dark. After green leaves have become yellow in the dark, formation of chlorophyll upon re-exposure of the plants to light is extremely slow. Colored substances other than chlorophylls *a* and *b* and carotenoids have not been found in these faded leaves or in pale green autumn leaves. Green algae and green leaves kept in the dark or taken from direct sunlight show no variation in their pigments (131, 138).

Due to the lability of the chloroplast pigments in killed cells and in solution, detection of traces of native chlorophylls in green tissues presents a difficult analytical problem. By a combination of adsorption and spectrographic methods, it has been shown that the amount of chlorophyll *b* in red algae or in diatoms could not have exceeded 0.3 per cent of the chlorophyll *a* (88), one part of chlorophyll *b* being readily detectable in the presence of 2,000 parts of chlorophyll *a* (140). Extension of these methods to easily extractable green algae and to higher plants now shows that these organisms can not contain even such small amounts of chlorophylls *c* and *d* and protochlorophyll.

All these observations indicate that chlorophyll formation and degradation are extremely complex, metabolic processes. Colored and magnesium-containing intermediates do not accumulate in significant quantities. By contrast, chlorophylls and carotenoids once formed within the living cells are remarkably resistant. Photosynthesis is not accompanied by formation of appreciable quantities of colored alteration products of the green or yellow pigments.

Chemical reactions of the phycobilins and of the carotenoids within the chloroplasts are even more obscure than those of the

chlorophylls. The carotenoids of all autotrophic plants, including seedlings, are formed in the dark, but the quantity of these pigments is usually increased in the light (113, 132). Precursors of the carotenoids have not been found, and little is known about the nature of the metabolic processes that lead to the formation of different carotenoids in various plants (170).)

NATURAL DISTRIBUTION OF PIGMENTS AND THE PRODUCTS OF PHOTOSYNTHESIS

Comparison of the pigments of autotrophic plants with the principal structural materials and reserve products (Table 6.2) reveals only one constant relationship; namely, the occurrence of chlorophyll *a* and the production of oxygen. No single pigment is invariably associated with another principal product of photosynthesis.

The chloroplast pigments themselves represent greatly different proportions of the photosynthetic products of various organisms. In vascular plants where the soluble products of photosynthesis are transported to large storage or structural organs, the amount of the pigments is very small relative to the total organic material produced. But in many algae where the products of photosynthesis are utilized quickly for the multiplication of cells, the chloroplast pigments may represent almost 10 per cent of the total organic matter. As indicated by sensitization reactions, remotely related plants form different proteins (90).

PHOTOCHEMICAL ACTIVITY OF DIFFERENT PIGMENTS

The absorption of energy by each of the pigments in plants depends upon the intensity and the spectral quality of the incident light, upon the spectral absorption properties and concentration of the colored constituents of the chloroplasts, and upon the geometrical arrangement or orientation of the pigments within the plastids. Under laboratory conditions, light of wave lengths absorbed principally by chlorophylls *a* and *b*, by phycobilins, and by some carotenoids is about equally effective in the photosynthetic process of a wide variety of plants: bacteria, diatoms, red algae, blue-green algae, and green algae (24, 28, 30, 54, 92).

In nature, most of the solar energy fixed by plants is probably absorbed by chlorophyll *a*. This is the most abundant chloroplast pigment with greatest absorption capacity for the intense yellow, orange, and red components of sunlight. In the narrow blue-green and blue spectral regions where absorption by carotenoid pigments is most significant, intensity of solar radiation decreases rapidly (93). Plants containing phycobilins, which absorb much of the incident green and yellow light, have a very restricted distribution.

Because of the considerable absorption capacity of water for red light, plants living in deep water receive a preponderance of blue-green light. Only in deep water are pigments other than chlorophylls likely to play a major role in the absorption and fixation of solar energy by autotrophic plants.

PIGMENTS AND THE MECHANISM OF PHOTOSYNTHESIS

Consideration of the various mechanisms of photosynthesis which have recently been critically reviewed by Manning, by Rabinowitch, and by Wassink is beyond the scope of this report. Suffice it to point out that the flow of energy into the photosynthetic process passes through a narrow channel, the pigment complex, composed principally of chlorophyll *a*. The course followed by most of this energy seems to be short and direct and leads to the production of oxygen. The course which leads to the reduction or fixation of carbon dioxide appears to be long and to have many by-ways.

The absence of alteration products of the chlorophylls in living plants exposed to light of different intensities (138) and the dissimilar nature of the several photosynthetically active chloroplast pigments indicate that absorption and transfer of radiant energy might not involve chemical reactions of the pigments (156). In view of the high efficiency of photosynthesis with varying amounts of the pigments, energy absorbed by each pigment molecule must be quickly and efficiently transferable into the photosynthetic process.

The occurrence of various chloroplast pigments in remotely related plants and the variation of the proportions of the pigments in related species grown under different conditions show that photosynthesis can not be attributed to any single combination or association of pigments. Similarly, the effectiveness of the spectral energy absorbed by different kinds of pigments indicates that the primary light absorption system is never a single chemical substance. From this point of view, the pigment-protein complexes extracted from disrupted chloroplasts might be more profitably regarded as parts of a microscopic organ, the chloroplast, rather than as chemical substances of constant composition. Conflicting views regarding the composition and function of these preparations have already led to a complex nomenclature; for example, chloroplastin, the substance from chloroplasts; and photosynthin, the photosynthetic substance from chemo-autotrophic bacteria.

Usually the lability of the photosynthetic system is ascribed to the instability of enzymes. Now it seems that the reactive units containing the pigments are fully as labile as enzymes.

Until more information is available one must consider the possibility that energy absorbed by the chloroplast pigments liber-

ates oxygen by a fairly direct reaction within the labile chloroplasts. The deoxygenated, reduced, or photolysed portion or product of the chloroplasts might subsequently react with water (112) to form a reducing agent capable of additional enzymatic reactions with various unsaturated organic materials such as carboxylic acids, carbonates, carbon dioxide, or nitrates with regeneration of the energy utilizing system.

As reported by Molisch and by Beijerinck nearly a half century ago, systems that liberate oxygen are difficult to isolate from living plants (42, 60). Mixtures of disrupted chloroplasts and ferric compounds, or quinones, liberate oxygen for short periods but without concomitant reduction of carbon dioxide (2, 56, 57, 80, 155). Mixtures of chloroplast material with dilute sugar solutions are also reported to liberate oxygen for a time (11).

In view of the fact that carbon dioxide is known to enter metabolic processes, including those of algae, of higher plants, and of animals, by a variety of chemical and enzymatic reactions, there may be many links in the metabolic chain connecting atmospheric carbon dioxide and the energy-absorbing apparatus of autotrophic plants. Elucidation of all these reaction systems is far from accomplishment.

OCCURRENCE OF PIGMENTS AND EVOLUTION OF THE PHOTOSYNTHETIC APPARATUS

PIGMENTS AND PHYLOGENETIC RELATIONSHIP

All the autotrophic plants that have survived the long course of evolution contain remarkably similar photosynthetic systems. The universal occurrence of chlorophylls and carotenoids in all autotrophic and chemo-autotrophic plants points to a common origin for these systems and possibly for the organisms as well.

Minor variations of the chlorophylls and xanthophylls have occurred with evolution of various taxonomic groups. Restriction of particular pigments to plants of one or of a few major groups indicates that variation of the pigment mixture must have occurred early in the evolution of the major taxonomic groups and before development of the species known today.

The occurrence of identical chloroplast pigments in most green algae, in ferns, mosses, and higher plants points to a close relationship among all these organisms, a view long held by phylogeneticists. The presence of similar xanthophylls and chlorophylls in diatoms and brown algae suggests a close phylogenetic relationship among organisms of these two groups. With respect to the occurrence of chlorophyll *c*, there seems to be a relationship among dinoflagellates, diatoms, and brown algae.

The great variation in the amounts of the phycobilins in closely related organisms indicates that these pigments may never have been indispensable constituents of the photosynthetic apparatus. The significance of phycobilins in phylogenetic relationships is not clear (138).

AGE OF THE PHOTOSYNTHETIC APPARATUS

The occurrence of identical pigments in such diverse groups as higher plants and green algae indicates that the common ancestors of these organisms must also have contained similar pigments. Since fossil green algae and rudimentary higher plants have been found in geological formations that are several hundred-million years old, the pigments of these kinds of organisms must have varied little, if any, during this long period.

Algae of the major classes probably existed in the Cambrian period. As pigments of organisms of each class are now relatively constant, there must have been few changes in the kinds or the proportions of the photosynthetically active pigments in hundreds of millions of years.

CHROMATIC ADAPTATION AND THE BATHYMETRIC DISTRIBUTION OF ALGAE

The common occurrence of red algae in deep water and the restriction of many green algae to surface waters led to the attractive and widely accepted theory of chromatic adaptation. Deep-water plants, which receive a preponderance of blue-green light, are presumed to have developed pigments, such as the phycobilins, whose color is complementary to the incident radiation. Absorption of blue-green light by these pigments also complements the weak absorption of this light by chlorophylls and supposedly enables the adapted organisms to live in the weak blue-green light at greater depths than the unadapted algae (30). An essential requirement of this theory is a high photosynthetic activity of light absorbed by complementary pigments, an effect first demonstrated convincingly by Englemann a half-century before the extensive investigations quoted in the preceding sections.

Gradual accumulation of evidence has shown many facets to this theory of chromatic adaptation. Owing to the significant spectral absorption capacity of chlorophyll *a* for blue-green light, particularly at the high concentration of the pigment in the plastids (138), the presence of complementary pigments may never have been essential for survival of plants in deep water. As pointed out already, adaptation of the pigment system through formation of complementary pigments does not now take place in response to variation of the spectral quality of the incident radiation. Although growth in deep water may have been a factor in the elimination

of plants that produced useless pigments, there is little indication that complementary pigments are formed in response to growth in deep water (138).

Recent unpublished results show that distribution of aquatic plants with respect to depth is not dependent upon the pigment composition of the organisms. Some of the green algae, Siphonocladiales, for example, which are found at the greatest depths inhabited by plants, contain the same pigments found in land plants. Some of the red algae and most of the blue-green algae are confined to shallow water or surface soil even though they contain large quantities of complementary pigments. As these plants do not lose their complementary pigments when growing in white light, there is no indication that the so-called chromatic adaptation is reversible.

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7.

The Photochemistry of Chlorophyll¹

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THE GOAL of all photosynthetic research is to interpret the process of photosynthesis which occurs in a living plant in terms of a series of individual steps of purely physical or chemical nature. Photosynthesis is a photochemical reaction, and like all such processes is made up of primary and secondary acts. The primary acts consist of the absorption of the photon by the chlorophyll molecule and of those simple reaction steps which follow thereupon immediately, and which involve only the molecule of chlorophyll and its immediate environment. The secondary steps are those chemical and physical processes which lead to the formation of the final chemical products.

The majority of photosynthetic researches has been devoted to secondary steps, in an attempt to elucidate the complex sequence of reactions which lead from the primary reactants, carbon dioxide and water, to the final products which are built up in the plant. Most biologists appear to believe that physicists and photochemists have solved the problem of the primary act. This would be true if we were dealing with atoms and diatomic molecules only. The exact nature of the primary act involving complex molecules is not well understood, even for relatively simple cases. It will be necessary, therefore, for us to study the optical and photochemical properties of chlorophyll and related compounds so that we can predict with some certainty what reactions can possibly constitute the primary process in photosynthesis.

The little which is known about the spectroscopy of chlorophyll can be represented in general outline by an energy level diagram (6), Figure 7.1. In this diagram the electronic levels are represented by the several horizontal lines, each of which is capped by a scroll

¹The experimental work upon which part of this review is based was performed under ONR Contract Noori-212, T.O. I at the University of Minnesota.

to indicate a continuum of overlapping oscillational energy levels. The arrows in this diagram represent transitions. Arrow 1 corresponds to a transition from the ground state to an oscillationally excited level of the first singlet excited state; that is, to the act of absorbing a quantum of red light. Since the excited chlorophyll molecule is surrounded by and in multiple collision with solvent molecules, it quickly loses its energy of oscillation to the solvent.

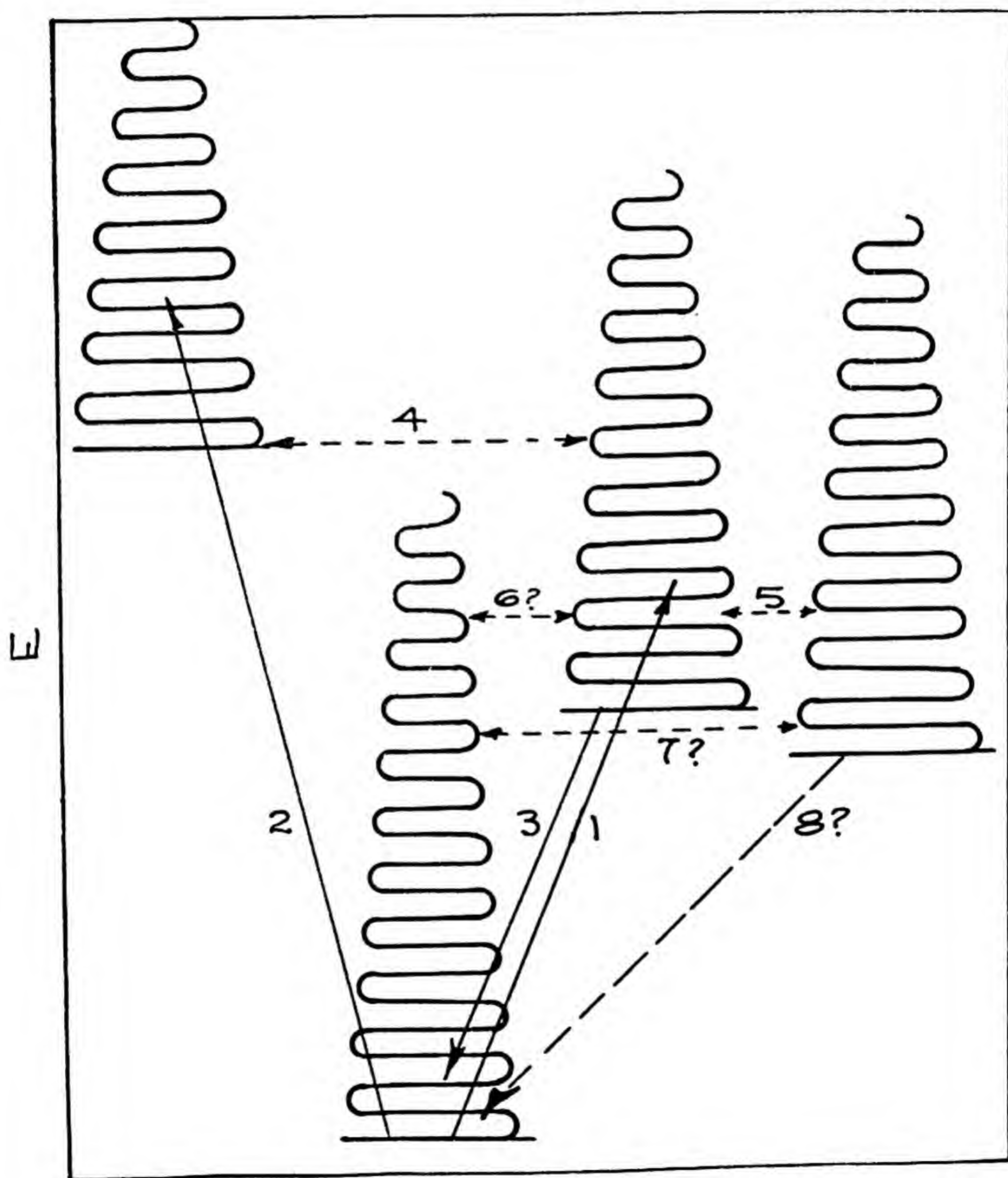


FIG. 7.1—Schematic potential energy diagram for the chlorophyll molecule.

Since the time required for the degradation of this oscillational energy is much shorter than the mean life of the electronically excited state, any fluorescence which the molecule exhibits will correspond to the transition from a low oscillational energy level of the excited state.

Transition 2 is similar to transition 1, except that it leads to the second electronic excited level and corresponds to the absorption of a quantum of blue or violet light. As is generally known, the electronically excited chlorophyll can lose its energy by fluorescence and this process is represented by arrow 3. Chlorophyll, similar to other pigment and dye molecules, does not exhibit fluorescence corresponding to the absorption act 2. When blue light is absorbed by the chlorophyll molecule only red light is emitted as fluorescence. The efficiency of the process of fluorescence for chlorophyll is low. It is estimated that only about 10 per cent of the absorbed light is re-emitted as fluorescence. The remainder of the absorbed energy must appear either as chemical energy, if reactants are present, or merely as heat if chlorophyll is dissolved in an inert solvent. The process by which the energy is degraded is sometimes called (21) *internal conversion*.

If only these two processes, step 3, fluorescence, and step 6, internal conversion followed by stepwise degradation of the energy, could occur, it would be impossible to explain the high yield of photosynthesized reactions which have been obtained by Gaffron (4) and others (5, 14, 15). It is necessary (3), therefore, to assume that there must be some long-lived state of the chlorophyll molecule and that practically all of the excited molecules, which do not fluoresce, go by a radiationless transition (internal conversion) to this long-lived state. This latter step is represented in the diagram by arrow 5. In some other complex molecules (11), although not in chlorophyll, it has been shown that the long-lived excited form of the molecule is a triplet state. In many cases (7), at low temperatures and in rigid solvents, phosphorescence (which is the reverse of step 5 followed by step 3) and long-lived fluorescence (step 8) of the molecule have been observed.

AVAILABLE PHOTOCHEMICAL METHODS OF EXPERIMENTATION

In principle there appear to be at least four ways by which we could obtain information regarding the primary acts of photosynthesis. One of these is to study, in homogeneous solution, the detailed kinetics of photochemical oxidation-reduction reactions sensitized by chlorophyll. While this is probably the most direct method of attack, it is unfortunately time-consuming.

Another method of studying the problem is to measure the

intensity of fluorescence of the chlorophyll molecule as a function of the nature of the solvent, of the temperature, and of the presence and concentration of various quenching agents. If we could be sure that the chemical properties of the singlet excited state and the long-lived excited state of chlorophyll molecules were the same, this would be an ideal method. In any case, it will give us valuable information about the formation of complexes between chlorophyll molecules and those of added materials. We may also hope that it will yield some information about the effect of the solvent on the ground state and perhaps upon the excited state of the chlorophyll molecules.

Studies of the effects of these variables upon the intensities of phosphorescence of chlorophyll would be indeed valuable. Unfortunately, the phosphorescence of chlorophyll, if it exists at all, must have such short life and such a low phosphorescence yield or such extremely long wave length that it is impractical to measure it. Two reports in the literature cite the appearance of the phosphorescence of chlorophyll. Kautsky (9), who worked with liquid solutions at ordinary temperatures, claims that a weak phosphorescence in the visible region, of a very short life, was observed in the complete absence of oxygen. Careful attempts to repeat this work by Dr. Linschitz (12) at the University of Chicago yielded only negative results. Calvin and Dorough (1) state that chlorophyll, dissolved in rigid solvents, at liquid nitrogen temperatures, phosphoresces with a mean life of about one-tenth of a second. Experiments performed in this laboratory (22) have shown that if there is phosphorescence under these later conditions, it must be either extremely weak or have a wave length greater than 9,000 Å or have a half-life of less than one-hundredth of a second. We have, therefore, been unable at present to use the study of phosphorescence as a method of obtaining information about possible primary reactions in photosynthesis.

Fortunately, chlorophyll has a unique property. In the absence of oxygen it undergoes what is sometimes called (17) reversible photobleaching. Its solutions, when free from oxygen, are reversibly bleached when they are illuminated by intense visible light. The extent of this reversible photobleaching under various environmental conditions appears to parallel other photochemical properties of chlorophyll, and to offer a reasonably direct method of investigating some of the chemical properties of the long-lived excited state.

REACTIONS PHOTOSENSITIZED BY CHLOROPHYLL

Although there have been many qualitative studies of reactions photosensitized by chlorophyll, only two such reactions have been investigated quantitatively. The first of these was the auto-oxidation

of allylthiourea in acetone solution and was studied by Gaffron (4) in 1927. His study is of importance chiefly because it demonstrates that chlorophyll can photosensitize an oxidation-reduction reaction with a quantum yield which approaches unity under favorable conditions. These measurements also indicate that the quantum yield decreases as the ratio of the chlorophyll to substrate concentration increases. This reaction, like the great majority of those which were studied qualitatively (19), involves molecular oxygen as the oxidizing agent. It is probable that an interaction of the long-lived excited state of the chlorophyll molecule with molecular oxygen is involved in all of these cases.

A reaction of another type, which was first investigated by Ghosh and Sen Gupta (5), is the oxidation of phenylhydrazine by the azo dye, methyl red. Further study of this reaction in this laboratory (15), has shown that the reaction occurs only in the absence of oxygen. If oxygen is present the photosensitized auto-oxidation of phenylhydrazine occurs in preference to the oxidation by methyl red. The detailed analysis of the kinetics of this reaction, which is published elsewhere (14), is consistent with the following mechanism.

1. $\text{GH} + h\nu \rightarrow \text{GH}^*$ absorption
2. $\text{GH}^* \rightarrow \text{GH} + h\nu_f$ fluorescence
3. $\text{GH}^* \rightarrow \text{GH}'$ internal conversion
4. $\text{GH}' \rightarrow \text{GH}$ degradation of the energy of
excitation
5. $\text{D} + \text{GH}' \rightarrow \text{DGH}$
6. $\text{DGH} \rightarrow \text{D} + \text{GH}$
7. $\text{PhH} + \text{DGH} \rightarrow \text{GH} + \text{DH} + \text{Ph}$
8. $2\text{DH} \rightarrow \text{D} + \text{DH}_2$
9. $2\text{Ph} \rightarrow (\text{Ph})_2$

In these equations the symbol GH stands for chlorophyll, GH^* for electronically excited chlorophyll, GH' for long-lived excited chlorophyll, PhH for phenylhydrazine, and D for (the intermediate form of) methyl red. While the data are not sufficient to determine this mechanism uniquely, they do eliminate many alternative possibilities.

It should be noted that, for the concentrations of chlorophyll used, there is no falling off of the quantum yield with increasing concentration of chlorophyll. This result makes it appear probable that the mechanism of this reaction is essentially different from that of the auto-oxidation of allylthiourea, even though the chlorophyll concentrations used in this study are at least tenfold less than those used by Gaffron (4). Measurements of the quenching of the fluorescence of chlorophyll by methyl red indicates that these molecules

form a complex with chlorophyll. It is quite likely that the reduction of methyl red involves this complex. Perhaps only light which is absorbed by the complex is able to bring about the reaction. The preceding mechanism can be readily modified to conform to this interpretation of the fluorescence quenching results without destroying its agreement with the kinetic data.

It is interesting that chlorophyll is unable to sensitize the reduction of methyl red when the reducing agent is allylthiourea, hydroquinone, or a number of other ordinary reactive reductants (14, 15). It is a curious characteristic of this reaction that of the three colored forms of methyl red, only that form which exists in the intermediate acid range is capable of undergoing the photosensitized reaction. The study of this reaction and of analogous processes will have to be greatly extended before we will be in a position to make any generalization about the mechanism of reactions photosensitized by chlorophyll.

THE FLUORESCENCE OF CHLOROPHYLL SOLUTIONS

The fluorescence spectrum of chlorophyll was studied very precisely by Zscheile (25) and by others (20). The absolute yield of fluorescence of chlorophyll solutions has been estimated (18) to be about 10 per cent. Until recently there has been little attempt to make a systematic study of the effects of solvent, temperature, and quenching agents upon the intensity of the fluorescence of chlorophyll. Dr. Watson (22), working in this laboratory, has begun such a program and has obtained the following results. In solvents such as ethyl ether, acetone, methanol, and ethanol, the fluorescence yield of chlorophyll is only slightly influenced by changing the solvent. However, if a pure hydrocarbon such as benzene, iso-octane, or normal heptane is used, the fluorescence of the chlorophyll is very much less than that obtained in the more polar solvents. If 10 per cent is the correct value for the fluorescence yield in methanol, the maximum yield obtainable in pure hydrocarbon is probably about one-half of 1 per cent.

The absorption spectrum of chlorophyll in dry hydrocarbons differs from that obtained in ordinary hydrocarbon solvents (26). In the dry solvents the maxima are lowered (without being shifted), and the detailed structure is eliminated. However, only a very small quantity of hydrogen-bonding impurity, such as methanol or water, need be present in the hydrocarbon in order to raise the fluorescence efficiency of the dissolved chlorophyll to as high a level as it has in pure methanol.

Figure 7.2 illustrates the effect of changing the methanol content in chlorophyll solutions in *n*-heptane. It is interesting to note that

in heptane or benzene containing a few hundredths of a per cent of methanol, the fluorescence efficiency of chlorophyll is slightly higher than it is in pure methanol. Studies of the effect of temperature upon the fluorescence yield in hydrocarbon solvents indicates that the low fluorescence is not due to dimerization of chlorophyll in the solvent. It seems possible, therefore, that the normal (10 per

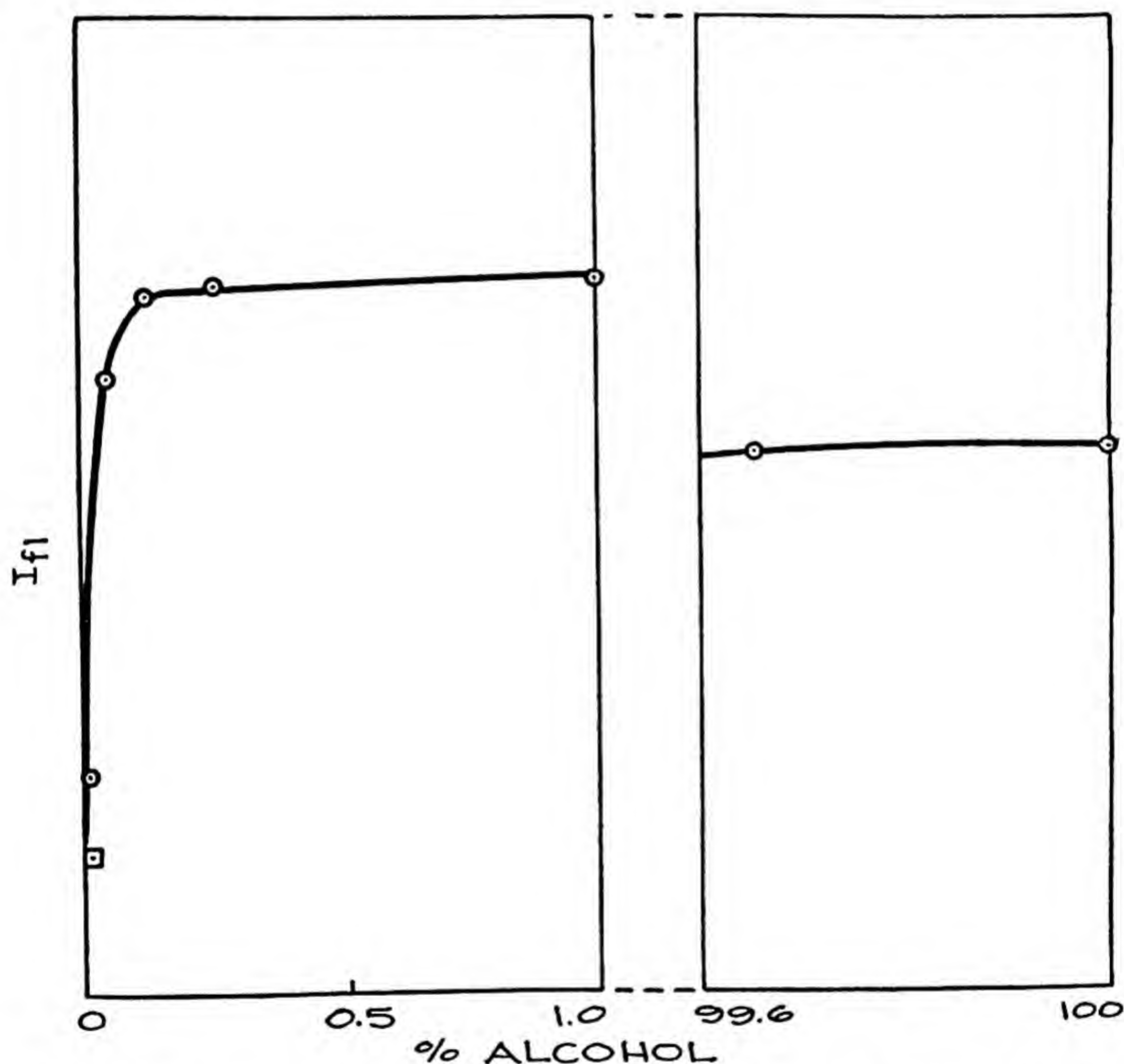


FIG. 7.2—Fluorescence of chlorophyll *a* in n-heptane—methanol mixtures.

cent) fluorescence is characteristic, not of the simple chlorophyll molecule, but of a solvated chlorophyll molecule containing one or more methanol or water molecules.

Phenylhydrazine, when added to solutions of chlorophyll in methanol, acts as a simple quenching agent (Fig. 7.4). However, its effect upon the fluorescence of chlorophyll in dry n-heptane is more complex. As is shown in Figure 7.3, low concentrations of

phenylhydrazine promote the fluorescence, increasing it to a maximum value about five times as great as that observed in the pure solvent. At high concentrations in the hydrocarbon solvent, phenylhydrazine acts as a simple quencher, following the Stern-Volmer law.

It is hoped that further study of these environmental factors upon the efficiency of the fluorescence of chlorophyll will yield informa-

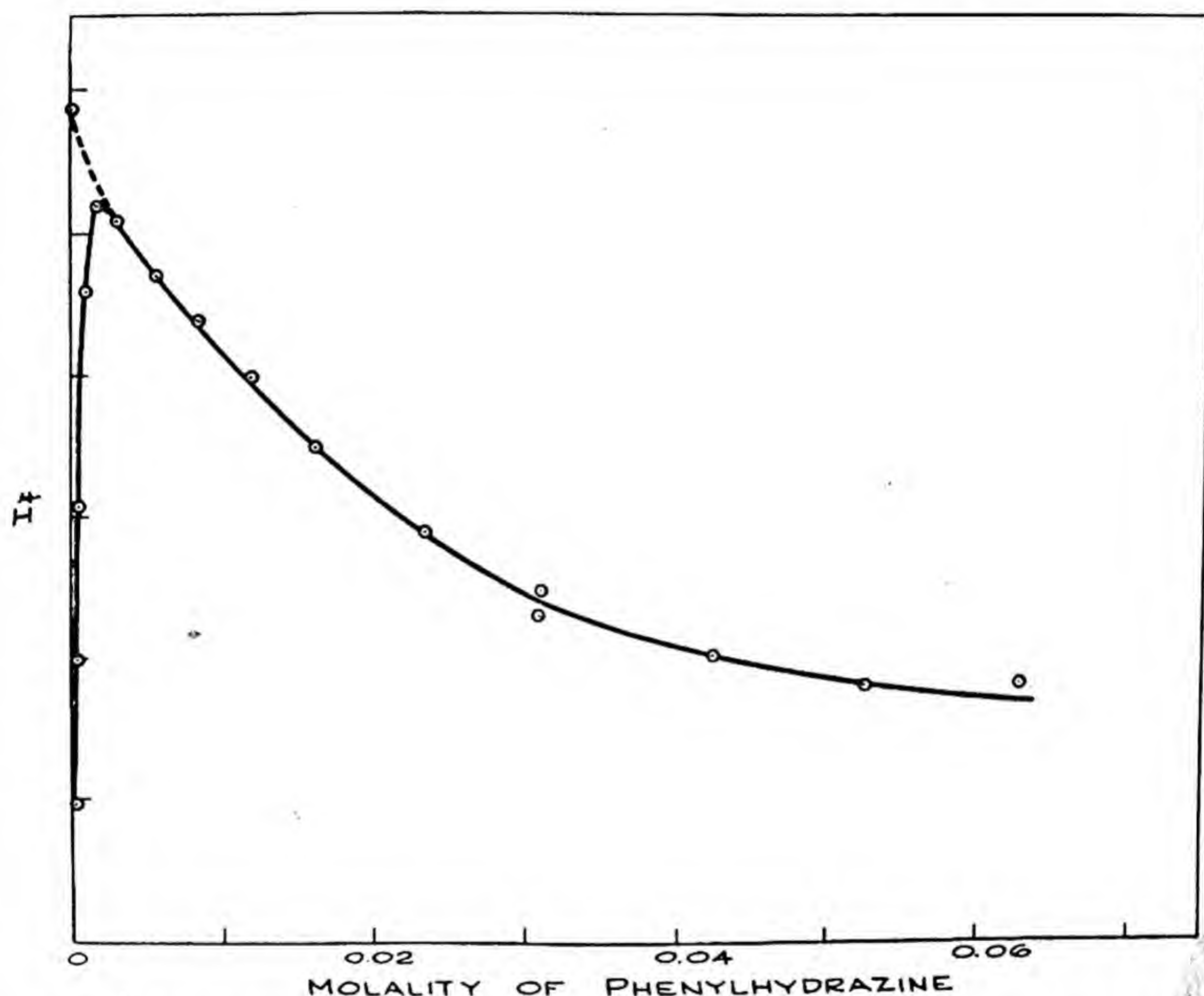


FIG. 7.3—Effect of phenylhydrazine upon the intensity of the fluorescence of chlorophyll *a* in benzene.

tion useful in interpreting the well-known fact that the fluorescence of chlorophyll in the intact plant is less than 1 per cent. There is no experimental evidence which supports the commonly held belief that photosensitization and fluorescence are complementary actions.

The fluorescence of chlorophyll in methanol or similar solvents is quenched (22) by a number of added substances. Among these are oxygen, phenylhydrazine, methyl red, and iodine. Typical reactive reducing agents, such as allylthiourea and hydroquinone, have little or no effect (2, 3, 22) upon the fluorescence of the chloro-

phyll. There appear to be two distinct types of quenchers, those which give a quenching action following the familiar Stern-Volmer law, and those which indicate that a compound is formed between the chlorophyll and the quenching agent. Oxygen is typical of the first group.

There are three lines of evidence indicating the occurrence of complex formation. First, there is a departure from the Stern-

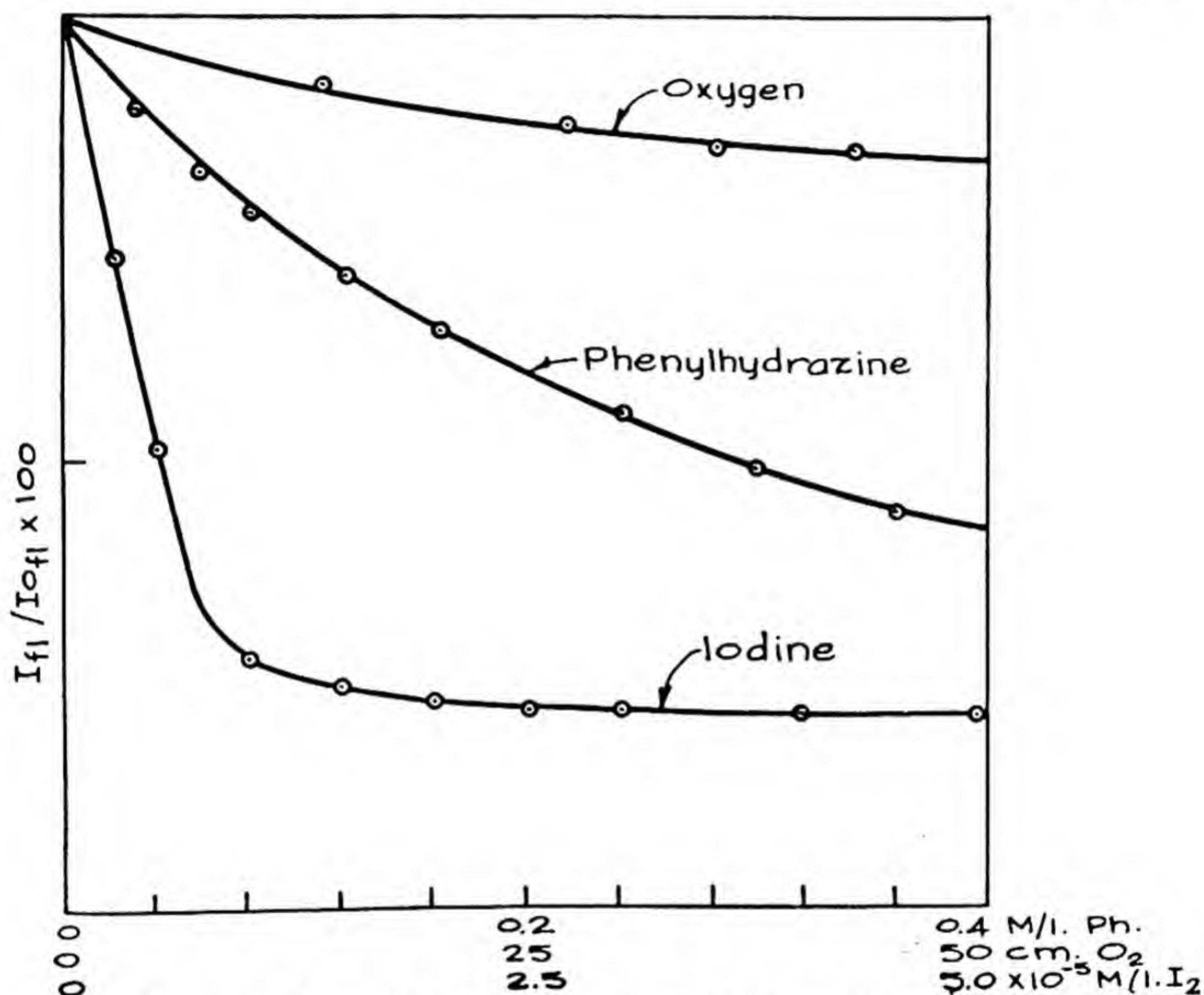


FIG. 7.4—Quenching of the fluorescence of chlorophyll *a* in methanol by phenylhydrazine, oxygen, and iodine.

Volmer law; the fluorescent yield approaches, in some cases, a finite value at a moderate concentration of the quencher, which does not change as the quencher concentration is further increased. Second, the quenching action is not immediately established upon the addition of the quenching agent, but requires a measurable time before it reaches its maximum value. Third, there is often a distinct change in the absorption spectra of the chlorophyll solution. Iodine (22) exhibits all three of these characteristics.

In Figure 7.4, the curve marked iodine shows a change of the fluorescent intensity with the concentration of iodine. This is clearly not of the Stern-Volmer type. For sake of comparison, the quench-

ing action of oxygen and of phenylhydrazine are also shown in this figure. The quenching of these two substances follows the Stern-Volmer law. (There is other evidence which indicates that chlorophyll and phenylhydrazine do form a compound.)

In Figure 7.5, the change of fluorescent yield with time, for solutions of chlorophyll *a* and chlorophyll *b* in the presence of a

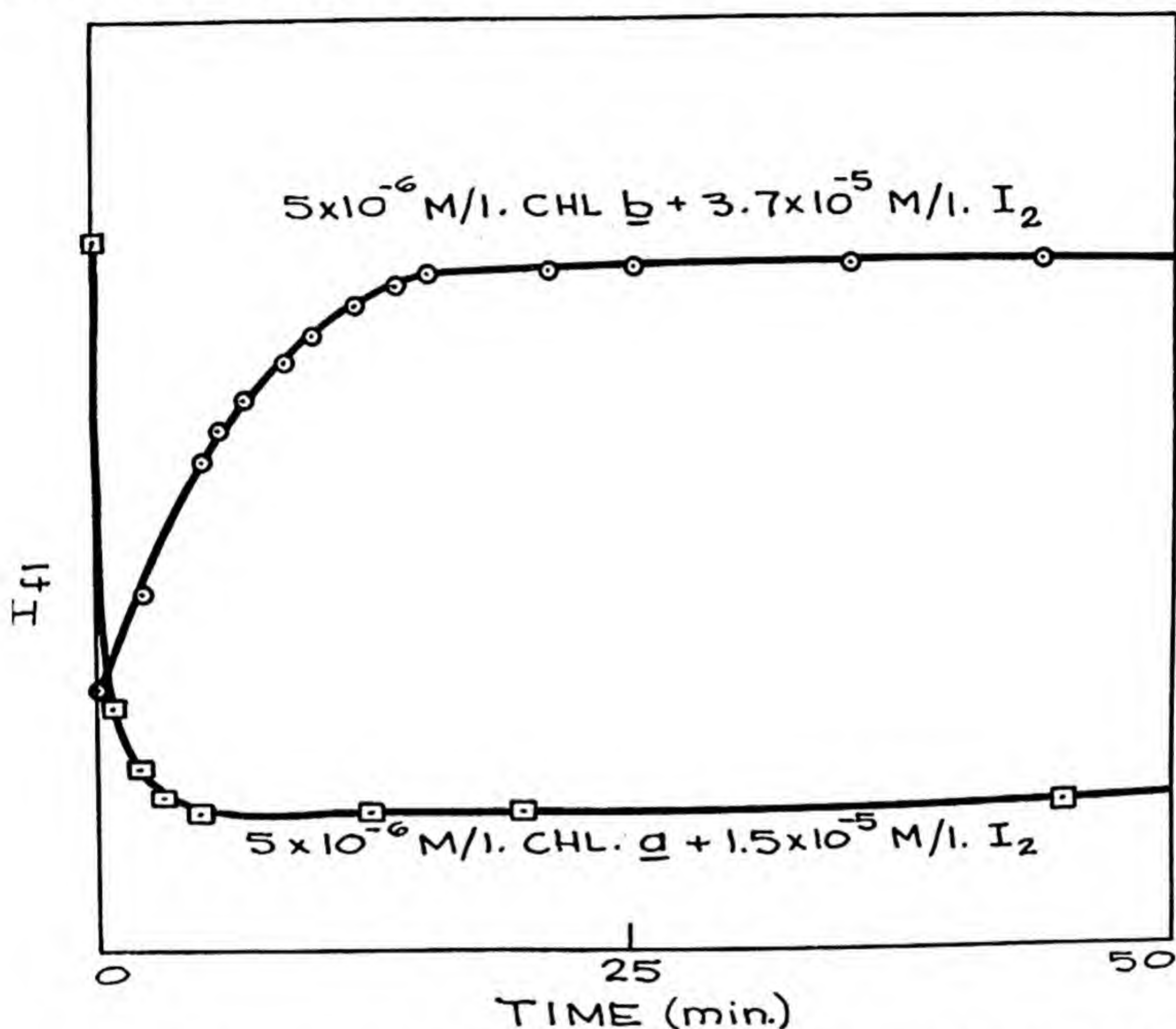


FIG. 7.5—The change of fluorescence of chlorophylls *a* and *b* with time in methanolic solutions containing iodine.

fixed amount of iodine, is illustrated. It is particularly interesting that the fluorescence of chlorophyll *b*, after a rapid drop which is not shown in the figure, gradually increases to a new flat value which is higher than the initial fluorescence of the chlorophyll *b* itself. The marked effect of iodine upon the absorption spectra of chlorophyll *a* and *b* is illustrated in Figure 7.6. While the change in spectrum of *b* is greater than the spectrum of *a*, the effect upon both of them is distinct and definite.

It has been reported by Weil-Malherbe and Weiss (24) that the fluorescence of chlorophyll in homogeneous solutions is very strongly

self-quenched. That is, normal chlorophyll molecules can degrade the energy of excitation of chlorophyll molecules in their first singlet excited state by a collisional process possessing very high efficiency. However, it has been shown recently (23) that this conclusion is due entirely to an incorrect interpretation of the measurements. The decrease in intensity of fluorescence which these authors

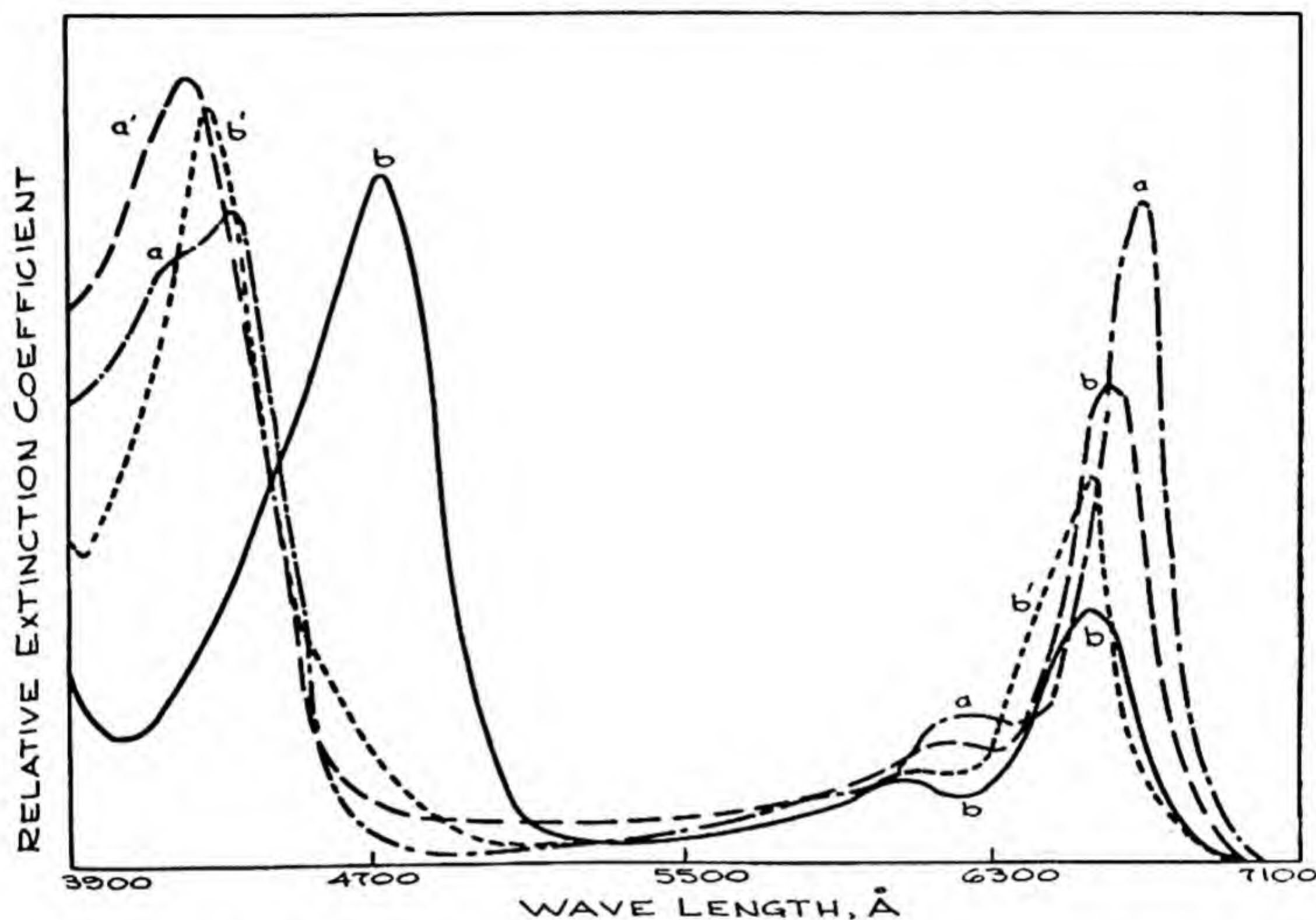


FIG. 7.6—The effect of iodine upon the absorption spectra of methanolic solutions of chlorophylls *a* and *b*; *a* = chlor. *a*; *b* = chlor. *b*; *a'* = chlor. *a* + $5 \times 10^{-3} M I_2$; *b'* = chlor. *b* + $2.5 \times 10^{-3} M I_2$.

observed was due to reabsorption of the emitted light by the chlorophyll. This criticism of the work of Weil-Malherbe and Weiss (24) could have been anticipated from the overlapping nature (25) of the absorption and fluorescent spectra of chlorophyll.

THE REVERSIBLE PHOTBLEACHING OF CHLOROPHYLL

Figures 7.7 and 7.8 (16) illustrate, respectively, the photobleaching of chlorophyll in methanol solutions in the presence and in the absence of oxygen. In the absence of oxygen the effect is almost completely reversible (10, 13, 16, 17). The extent of the bleaching is proportional to the square root of the light intensity. In the presence of oxygen the bleaching is completely irreversible. Both the rate of the irreversible bleaching and the extent of the rever-

sible bleaching are independent of temperature in the range from 0° to 30°C. (10). In methanol solutions containing a large excess of oxygen, the quantum yield of the irreversible bleaching is about 5×10^{-5} molecules of chlorophyll destroyed per photon absorbed. We can estimate that the quantum yield of the reversible bleach-

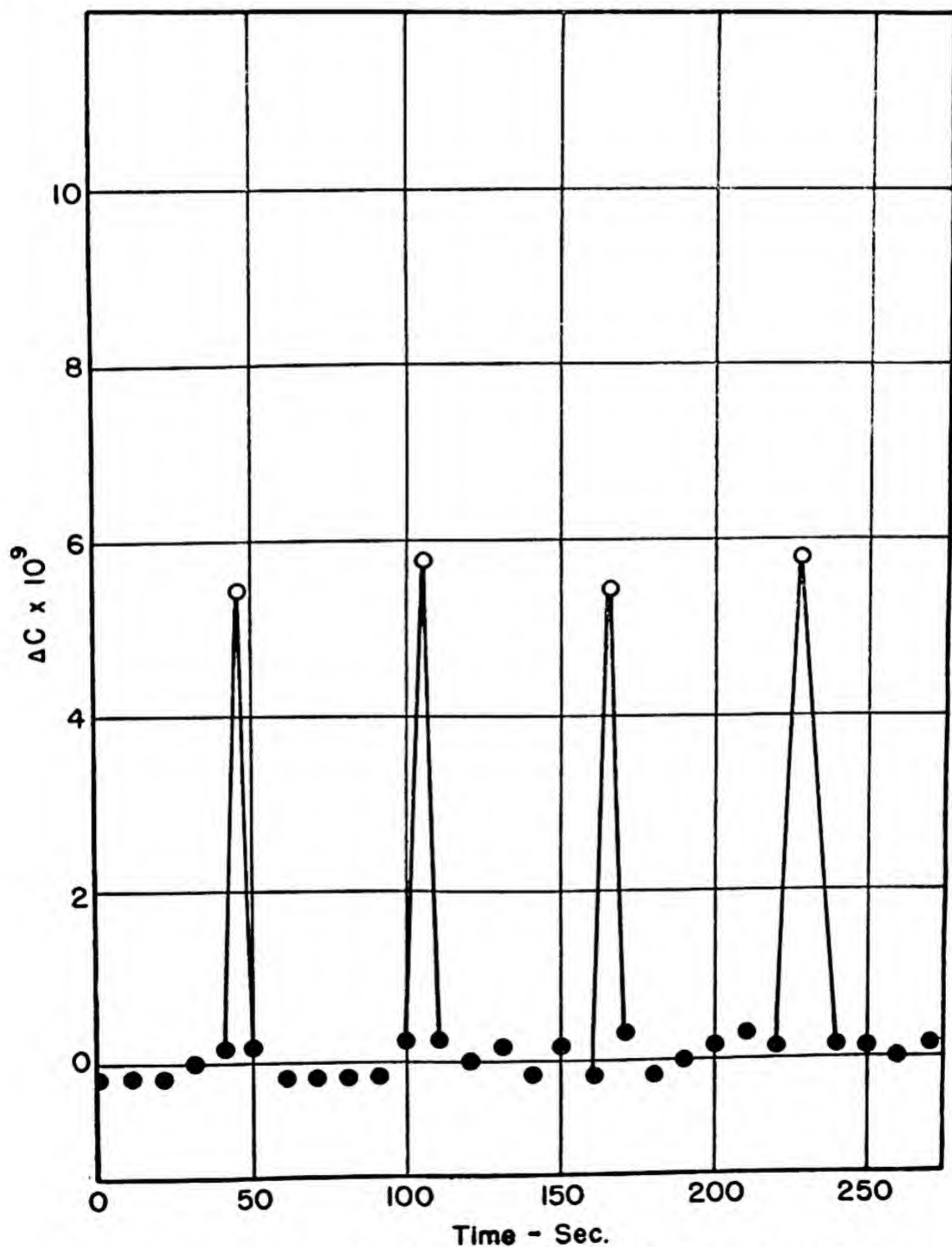


FIG. 7.7—Reversible photobleaching of chlorophyll in oxygen-free solutions.

ing in the absence of oxygen is at least thirtyfold greater. This shows, as first pointed out by Rabinowitch (17), that oxygen inhibits the reversible bleaching in addition to transforming part of it into an irreversible process. Most of the measurements have been made with solutions of chlorophyll *a*; however, both chlorophyll *a* and *b* exhibit the phenomenon to a comparable extent. There is some evidence (16) that chlorophyll *b* undergoes a slightly greater reversible bleaching and a lesser irreversible bleaching.

Solvent effects paralleling those which were obtained in the fluorescence measurements, have been observed (10) in reversible bleaching. If pure dry benzene is used as the solvent, there is no measurable reversible bleaching, and the irreversible bleaching is extremely small in the absence of oxygen. However, if 1 per cent of methanol is added to the benzene, the reversible bleaching is restored practically to its original value. A few measurements have been made with acetone and with a 50 per cent mixture of methanol and isoamylamine (13). These solutions show reversible bleaching similar to that obtained with pure methanol solutions.

The extent of the reversible bleaching is affected by the addition of small amounts of a number of substances. The changes in reversible bleaching produced by typical reagents are summarized in Table 7.1.

TABLE 7.1
THE EFFECT OF ADDED SUBSTANCES UPON THE REVERSIBLE
BLEACHING OF CHLOROPHYLL

Solvent	Added Substance		Approximate Ratio of the Steady-State Bleaching in the Solution to That in Pure CH ₃ OH	References
	Compound	Concentration		
CH ₃ OH.....	O ₂	10 ⁻⁵ M	0 (a)	10, 13, 16, 17
CH ₃ OH.....	Fe ⁺⁺	?	0	17
CH ₃ OH.....	allylthiourea	0.05M	1	13, 16
CH ₃ OH.....	hydroquinone	.01M	1	13
CH ₃ OH.....	isoamylamine	50%	1	13
CH ₃ OH.....	CO ₂	?	1	17
CH ₃ OH.....	H ₂ O	2%	2	10
CH ₃ OH.....	oxalic acid	10 ⁻⁴ M	3	16
CH ₃ OH.....	methyl red	10 ⁻⁵ M	4	16
CH ₃ OH.....	LaCl ₃	10 ⁻⁴ M	5 (b)	10
CH ₃ OH.....	CeCl ₃	10 ⁻⁴ M	10 (b)	10
CH ₃ OH.....	I ₂	10 ⁻⁵ M	150 (b)	10 16
(CH ₃) ₂ CO.....	none	1	13
C ₆ H ₆	none	0	10
C ₆ H ₆	CH ₃ OH	1%	1	10

(a) Irreversible photobleaching occurs.

(b) An irreversible thermal reaction occurs.

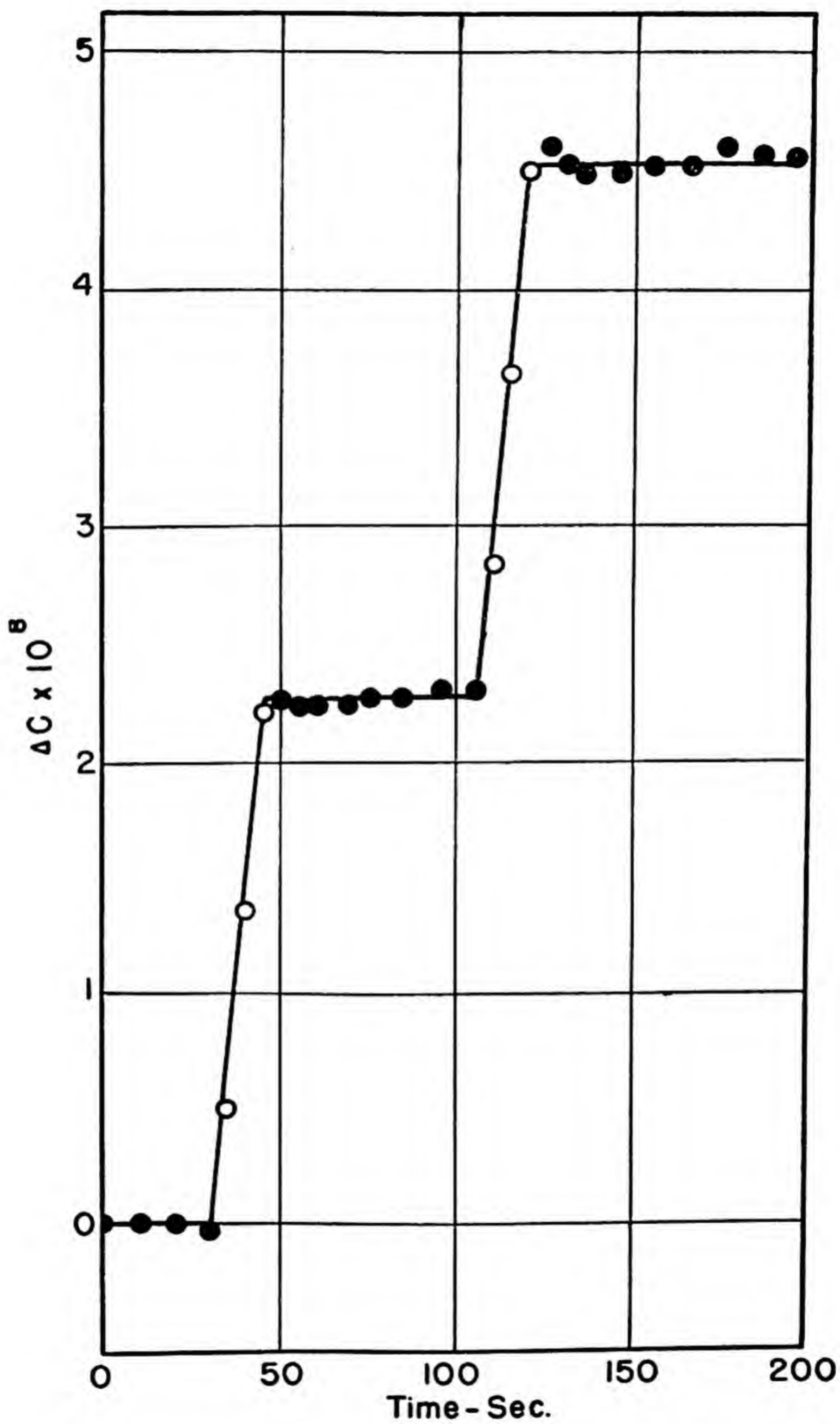


FIG. 7.8—Irreversible photobleaching of chlorophyll in solutions saturated with air.

It is noteworthy that some substances, like oxalic acid (10, 16) or iodine, not only greatly increase the extent of the reversible bleaching but also change the form of the rate law by which the bleached material returns to its original color. Again paralleling the results obtained in fluorescence quenching, the effect of iodine on reversible bleaching is particularly striking. It is somewhat surprising that presumably inert salts, like lanthanum chloride (10), cerous chloride, and to a lesser extent, barium chloride, increase

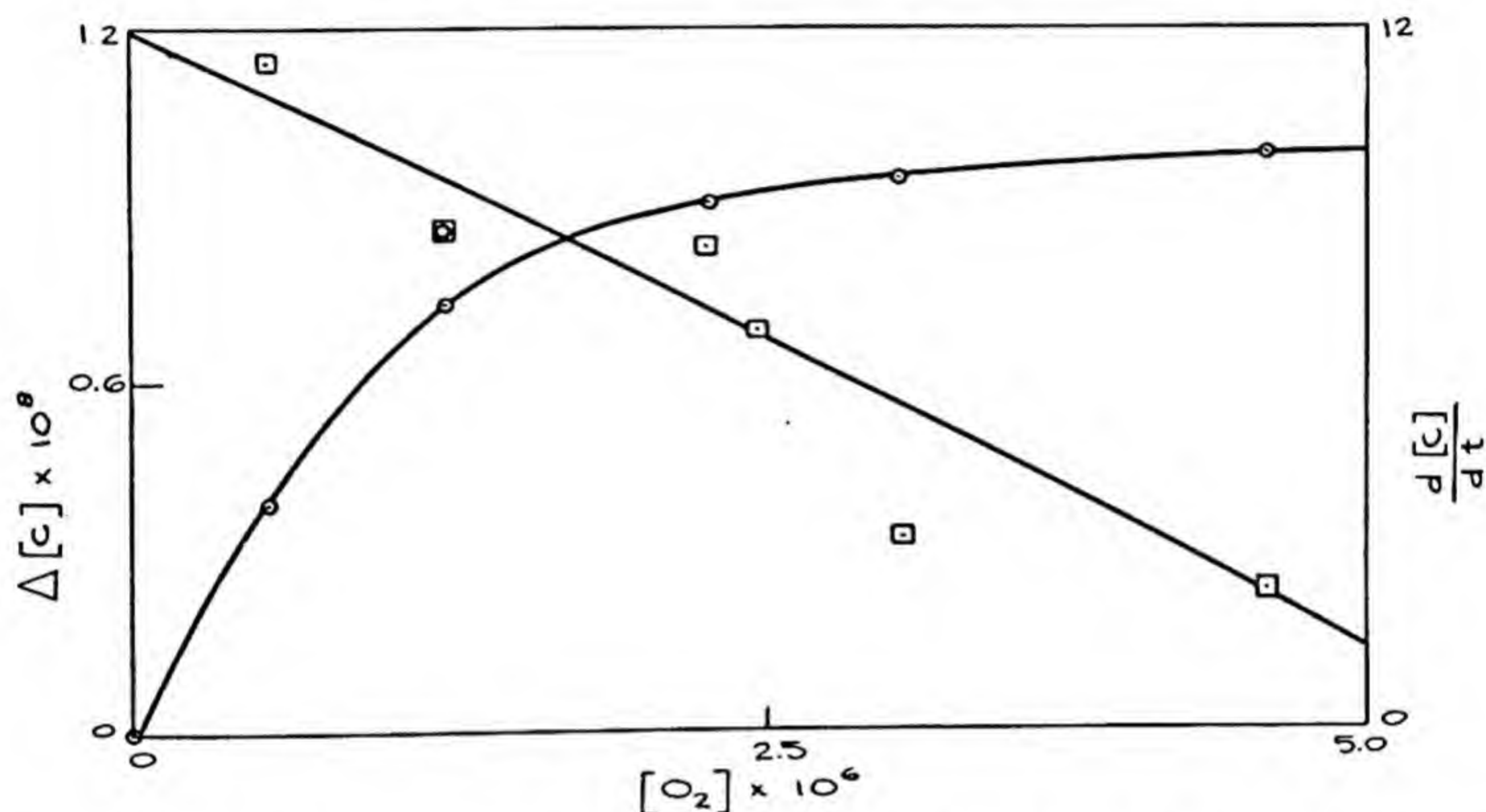


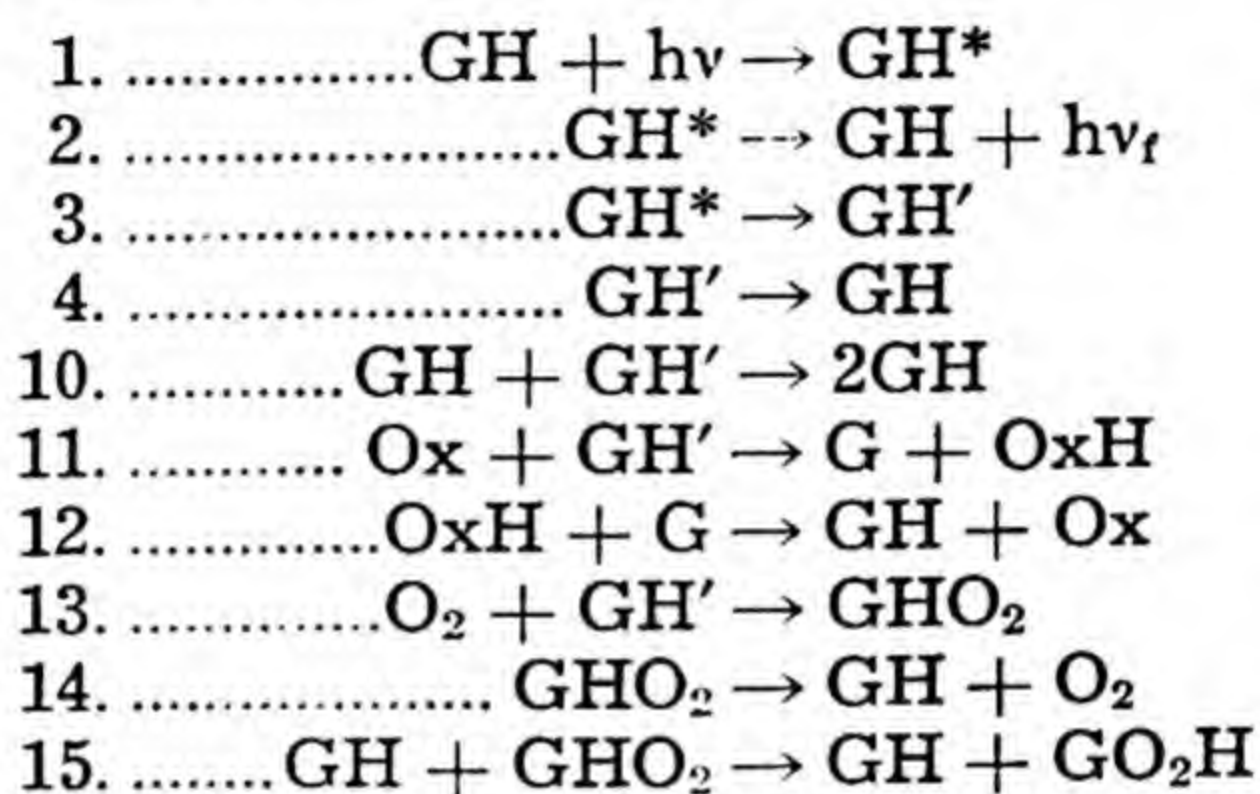
FIG. 7.9—Reversible and irreversible photobleaching of chlorophyll as a function of the molality of dissolved oxygen.

the extent of the reversible bleaching and slow down the rate of recovery.

At very low oxygen concentrations irreversible and reversible bleaching occur simultaneously. Figure 7.9 (10) illustrates the quantum yield of the irreversible bleaching and the maximum extent of reversible bleaching as a function of the concentration of oxygen in the range from the 0 to 5×10^{-6} molar. At this latter concentration practically the maximum effect of oxygen has been attained, and further increase in the oxygen content has little effect upon either the reversible or irreversible bleaching. Although the extent of the reversible bleaching as a function of oxygen concentration is indicated by a straight line in the diagram, this is perhaps more representative of the inaccuracy and difficulty of these measurements than it is of the actual relation between concentration and extent of bleaching.

The extent (absolute) of the reversible bleaching decreases slightly (10) as the chlorophyll concentration is increased from 1.0×10^{-6} to 5.0×10^{-6} molar. When the concurrent increase in the intensity of the absorbed light is taken into account, it appears that the extent of the reversible bleaching is inversely proportional to the square root of the chlorophyll concentrations.

The following equations represent a series of reaction steps which satisfactorily explain all of the major features observed in the reversible bleaching reaction. Furthermore they are consistent with information obtained from fluorescence quenching experiments and from a study of photosensitized reactions.



The symbols used in the first four equations have the meanings already stated. The new symbols have the following significance: Ox, an oxidizing agent present as a trace impurity in the solvent or possibly the solvent itself; GHO_2 , an unstable oxide of chlorophyll; and GO_2H , an irreversibly oxidized, bleached product of chlorophyll.

It is a necessary consequence of the present measurements that at concentrations of 10^{-6} molar or greater the degradation of the long-lived excited chlorophyll is not a spontaneous or unimolecular process but apparently goes by a bimolecular reaction with a normal chlorophyll molecule. A process similar to this has been suggested (8) as a possible explanation for the failure of most complex organic molecules to show phosphorescence in liquid solutions. This effect is also very probably closely related to Gaffron's (4) observation that the quantum yield of photosensitized auto-oxidation of allylthiourea decreases as the ratio of the concentration of chlorophyll to allylthiourea is increased.

SUMMARY

Although the systematic study of the photochemical properties of chlorophyll solutions has only begun, we are in a position to make a few generalizations of some interest. First, under favorable

conditions, chlorophyll is capable of photosensitizing oxidation-reduction reactions with a yield approaching unity (4, 5, 14). Second, such reactions are not limited to those involving oxygen (5, 15, 19) but may involve other oxidizing agents, such as azo dyes. Unlike auto-oxidations, chlorophyll-sensitized reactions involving azo dyes as oxidants are comparatively specific (14, 15) in that they will occur only if certain types of reducing agents are present. Third, chlorophyll apparently forms complexes (15, 22) with a number of substances, such as lanthanum chloride, iodine, methyl red, and possibly phenylhydrazine. Fourth, the photochemical properties of chlorophyll solutions depend to a great extent upon the solvent (5, 10, 22). Also, many of the properties formerly attributed to chlorophyll solutions in general appear to be peculiar to chlorophyll molecules in the presence of polar molecules of the hydrogen-bonding type. This is not a simple effect of the polarity of the solvent (22), since a very small addition of such solvents (as methanol) to a large excess of an inert hydrocarbon solvent has as great an effect as the complete substitution of the polar solvent for the inert one.

We can, in addition, draw certain conclusions about types of mechanisms which are apparently available for chlorophyll molecules. Perhaps the most important (3) is that it is not the directly excited singlet state of the chlorophyll molecule which is photochemically active but rather the indirectly-formed, long-lived state of the molecule. In homogeneous liquid solutions, it is also necessary to assume that these long-lived excited molecules disappear (8, 10), not by a unimolecular process, but by reaction with normal chlorophyll molecules. We can expect, therefore, that if experiments on photosensitization were carried out in a more viscous medium, one could go to higher concentrations of chlorophyll without reducing the quantum yield of the reactions. There is as yet no direct evidence to support the view that chlorophyll, in acting as a photosensitizer, is alternately oxidized and reduced. The belief that fluorescence and photosynthesis are complementary actions of chlorophyll in the chloroplasts, gains no support from *in vitro* experiments with chlorophyll or from photochemical measurements on other dyes or pigments.

It is certainly premature to attempt to apply these findings to the chemical and physical properties of the chlorophyll molecule in the chloroplast. Undoubtedly such relations exist, and it seems probable that when we have obtained more certain and detailed information about the chlorophyll molecule in simple homogeneous systems, we shall be in a position to restrict the possible reaction steps which the chlorophyll molecule can undergo in the chloroplasts.

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8.

Chlorophyll Studies

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THE COMPLICATED MIXTURE of pigments in the chloroplasts of green plants yields upon suitable extraction and separation two green substances, chlorophyll *a* ($C_{55}H_{72}N_4O_5Mg$) and chlorophyll *b* ($C_{55}H_{70}N_4O_6Mg$). In general, exposure of the plant to visual radiation is necessary for the formation of these pigments. Plants grown in the dark—etiolated plants—contain a number of yellow pigments and a small amount of a green material which is, however, spectroscopically different from chlorophyll and which has been called protochlorophyll. Chlorophyll formation is supposed to take place upon irradiation from protochlorophyll as precursor. The close chemical relationship of chlorophyll and protochlorophyll was established, and the partial synthesis of protochlorophyll was accomplished by Fischer (1) by introducing magnesium and phytol into vinyl pheoporphyrin a_5 . On the basis of our present knowledge the formula, Figure 8.1, represents the best expression of the structure of chlorophyll according to Fischer (2).

According to this formula chlorophyll has three asymmetric carbon atoms; however, the partial synthesis of methylpheophorbide *a* showed that carbon atom 10 does not function as a center of asymmetry, probably due to the ease with which the carbonyl group in position 9 is enolizable. Hence, only the carbon atoms in 7 and 8 positions are responsible for the optical activity of chlorophyll and its derivatives. For the numbering system used in formulae of chlorophyll and related compounds see Figure 8.4.

By the action of even very weak acids the complexly bound magnesium atom in chlorophyll is replaced by two hydrogen atoms to form pheophytin. Hydrolysis with strong acids removes the phytol group also, yielding pheophorbide. Pheophorbide *a* or *b* may be reconverted into the corresponding chlorophyll by esterifying the propionic acid side chain in position 7 with phytol $C_{20}H_{39}OH$ and introducing the magnesium atom by means of Grignard reagent.

If pheophorbide *a* is treated with hydriodic acid, the two hydrogen atoms at carbon atoms 7 and 8 in ring IV migrate onto the vinyl group on carbon atom 2. The resulting compound is pheoporphyrin *a*₅ (Fig. 8.8, VIII). In Fischer's nomenclature chlorophyll derivatives retain the letter of the chlorophyll component from which they are derived. The suffix indicates the number of oxygen atoms

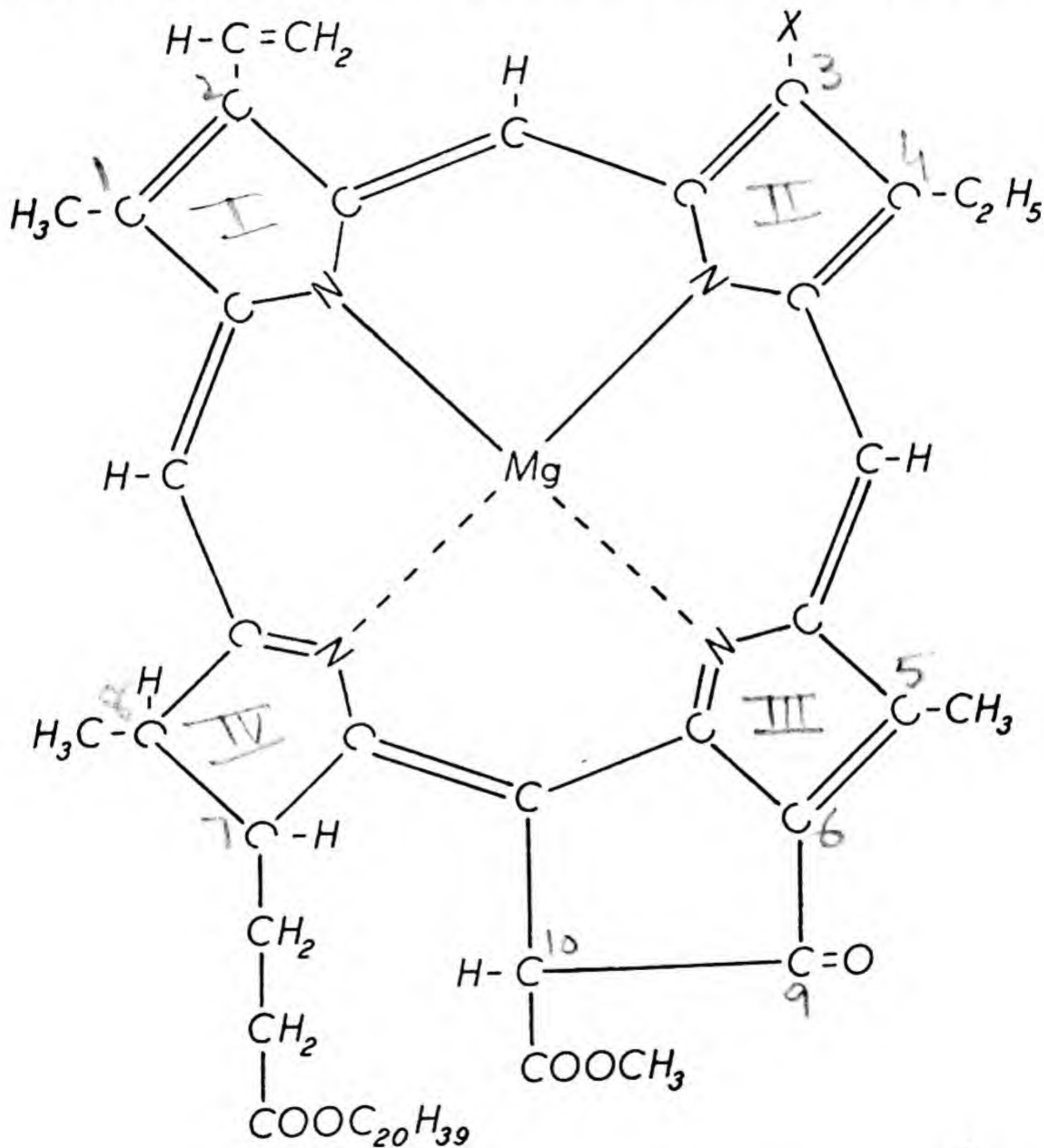


FIG. 8.1—In chlorophyll *a*, $X = -CH_3$. In chlorophyll *b*, $X = -C(=O)H$

in the molecule. A general review of chlorophyll, its derivatives, and their reactions may be found in an article by Rothemund (3).

Fischer's total synthesis of pheoporphyrin a_5 (4) is the closest approach to the synthesis of chlorophyll a thus far. In order to present briefly the essential steps of that synthesis, it is desirable to list first the fundamental ring systems important in chlorophyll

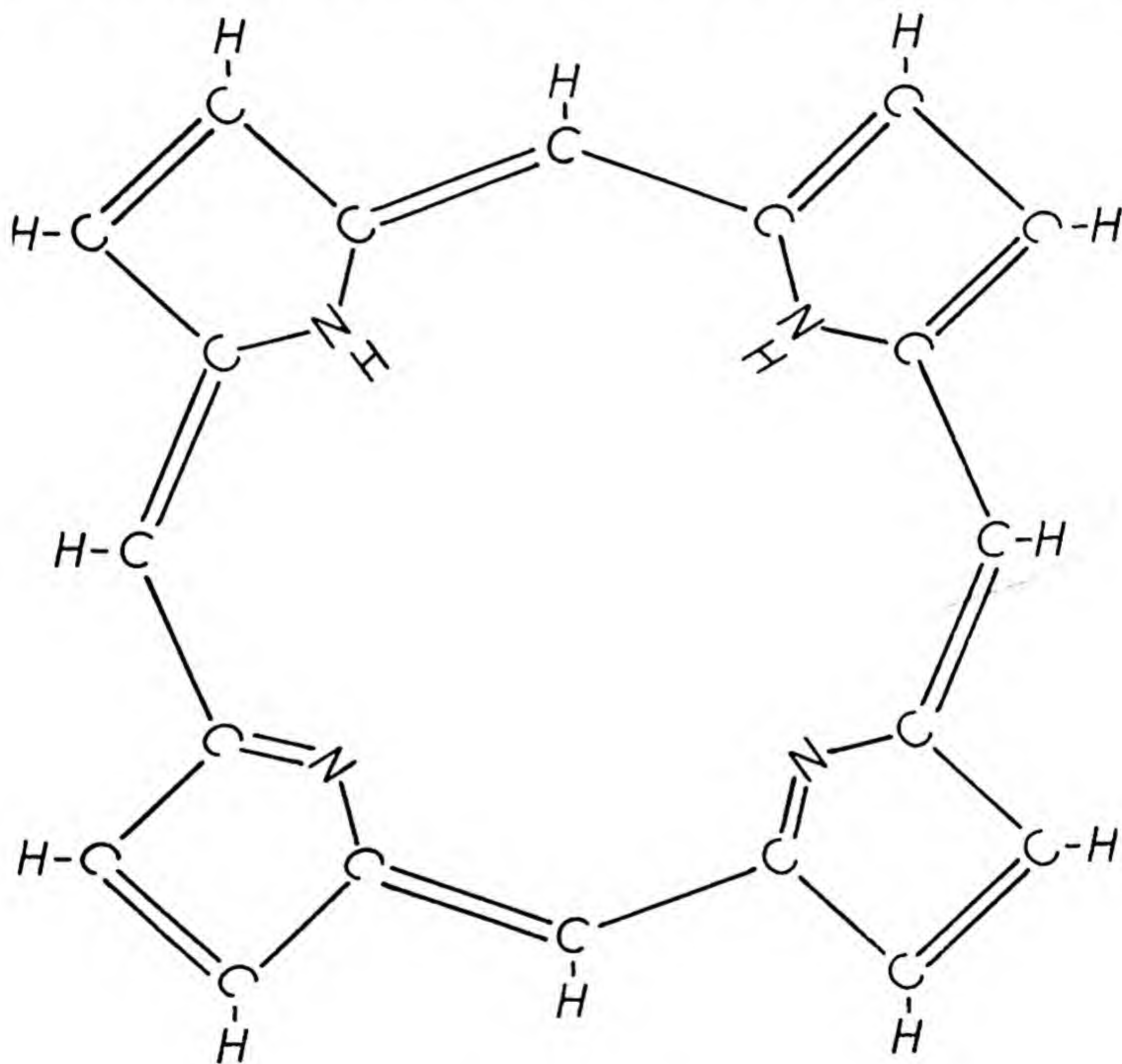
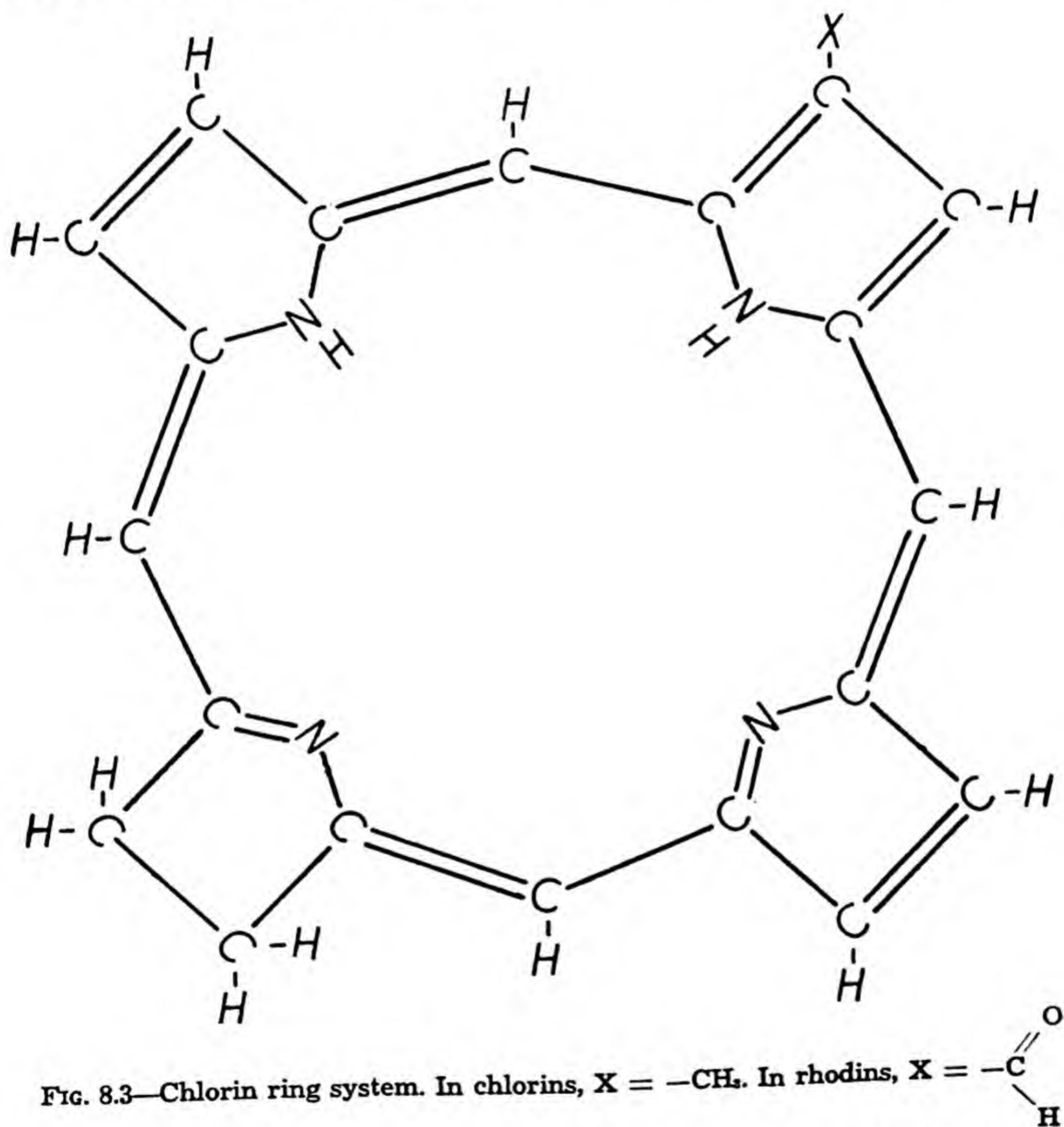


FIG. 8.2—Porphine.

chemistry. They are the systems of porphine (Fig. 8.2), chlorin (in the a -series), or rhodin (in the b -series) (Fig. 8.3), and phorbin (Fig. 8.4).

Of these fundamental ring systems only porphine has been synthesized; from pyrrole and formaldehyde by Rothemund (5)

and from pyrrole- α -aldehyde by Fischer and Gleim (6). The synthesis by Rothemund can be extended to combining pyrrole and other aldehydes and leads in this general form to $\alpha,\beta,\gamma,\delta$ -tetrasubstituted porphines, e.g., with pyrrole and benzaldehyde to $\alpha,\beta,\gamma,\delta$ -tetraphenylporphine (Fig. 8.5).



Substituted porphines are called porphyrins. Of the large number of possible porphine and porphyrin metal complex salts, the $>Fe^{++} - Cl$ complexes are referred to as hemins, the $>Mg^{++}$ complexes, as phyllins.

The phyllin of pheoporphyrin a_5 monomethyl ester (Fig. 8.6) is isomeric with chlorophyllide a (Fig. 8.7). A comparison of the

formulae of chlorophyll *a* (Fig. 8.1) and of chlorophyllide *a* (Fig. 8.7) with the formula of pheoporphyrin *a*₅ (Fig. 8.8, VIII) and its phyllin (Fig. 8.6) shows the following differences: in chlorophyll *a* and in chlorophyllide *a* the substituent in position 2 is the vinyl group $-\text{CH}=\text{CH}_2$, and two extra hydrogen atoms are

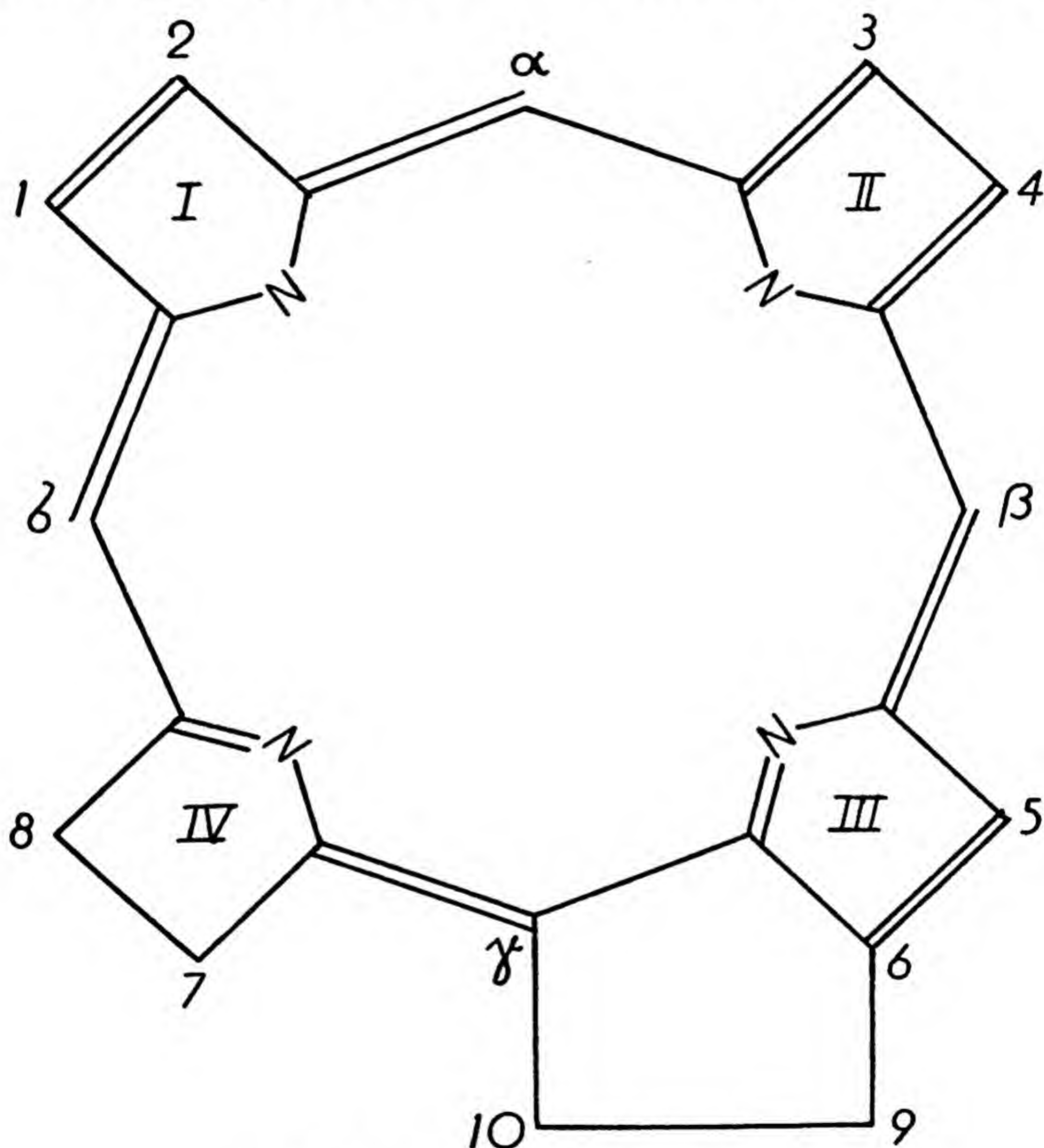


FIG. 8.4—Phorbin ring and nomenclature.

located in positions 7 and 8. In pheoporphyrin *a*₅ and in its metal complex salt the vinyl group is hydrogenated to the ethyl residue and a double bond exists between the carbon atoms 7 and 8. The

isomers differ, therefore, only in the location of one double bond; either it is in the substituent in position 2, or it is in ring IV between the β carbon atoms. For the purpose of structural considerations the phytol group esterifying the propionic acid residue

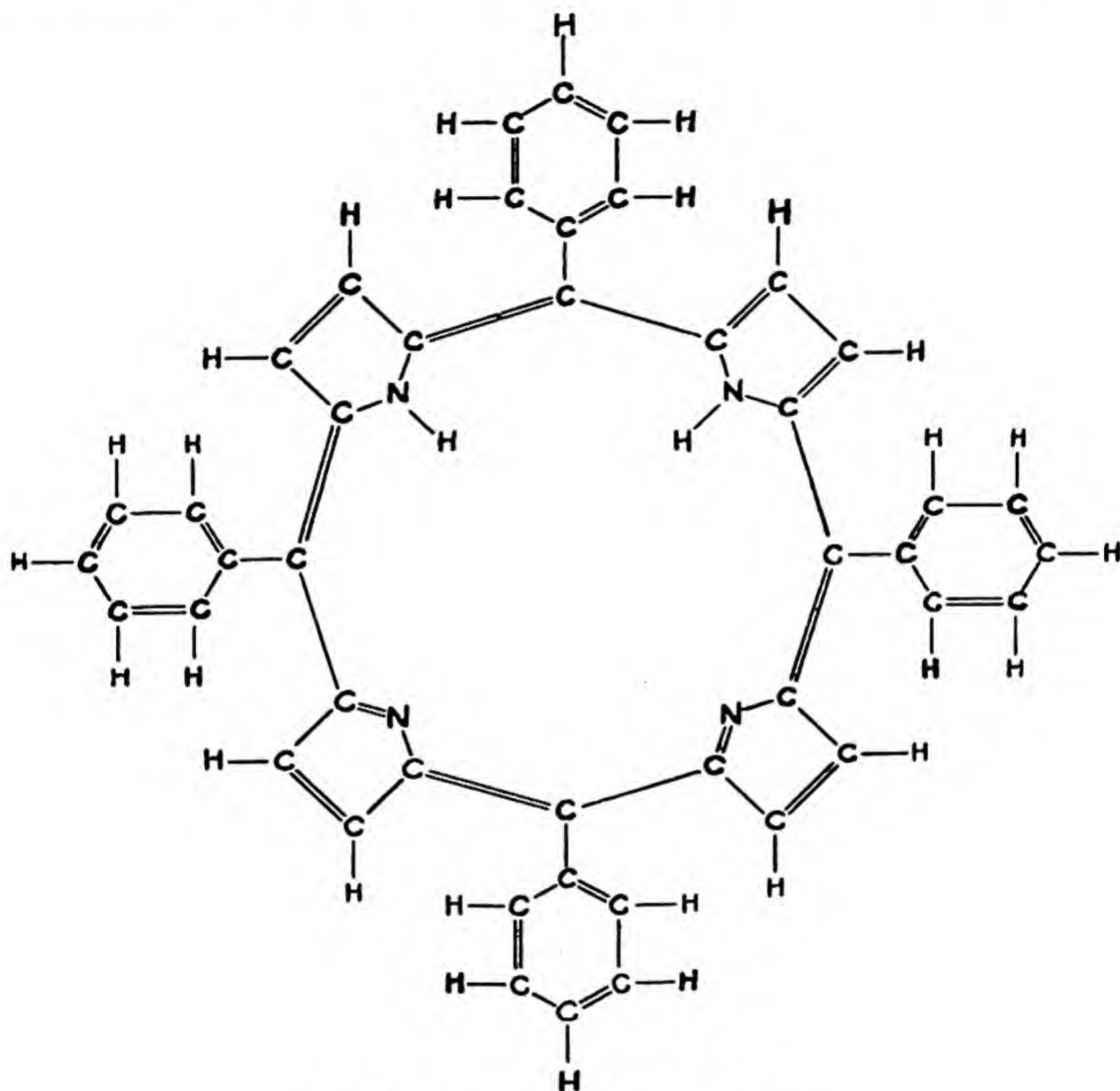


FIG. 8.5— α , β , γ , δ -Tetraphenylporphine.

in position 7 of chlorophyll *a* can be overlooked, since the removal and the introduction of this group can be easily accomplished by means of the enzyme chlorophyllase.

In synthetic work with pheoporphyrin *a*₅ the methods of direct synthesis of the entire ring system from dipyrromethenes (Fig. 8.9, I) with proper substituents failed because of the great sensitivity of this porphyrin to the usual reaction conditions. It was necessary to resort to chemical reactions which allowed modifications of the side chains after the ring had been synthesized. Thus

Fischer (4) accomplished the synthesis of pheoporphyrin a_5 by building up the carbocyclic ring from 6 to γ with its carbethoxy residue in position 10 as shown by the formulae in Figure 8.8.

Synthetic phylloporphyrin (Fig. 8.8, I) was the starting material.

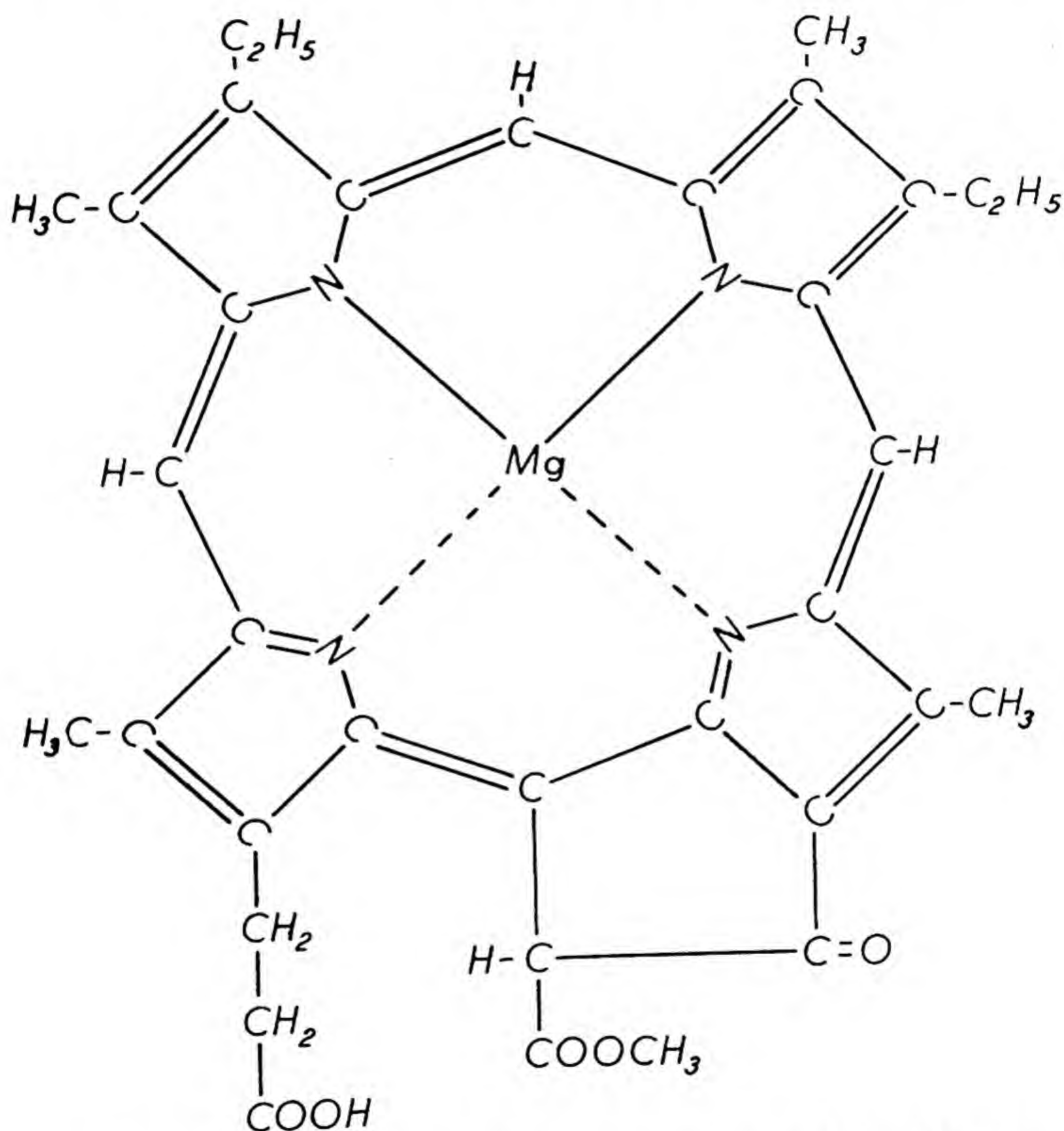


FIG. 8.6—Phyllin of pheoporphyrin a_5 monomethyl ester $C_{35}H_{24}N_4O_5$ Mg.

Its γ methyl group was changed by means of iodine into a formyl group, yielding γ -formyl pyrroporphyrin (II). With HCN the corresponding cyanhydrin (III) was formed, and from it on treatment with concentrated H_2SO_4 pyrroporphyrin- γ -glycolic

acid amide (IV) was obtained. This acid amide was catalytically reduced to the substituted acetic acid amide. From the latter substance iso-chloroporphyrin e_4 was produced by hydrolysis of the amide group. When the hemin ester of this porphyrin (V) was

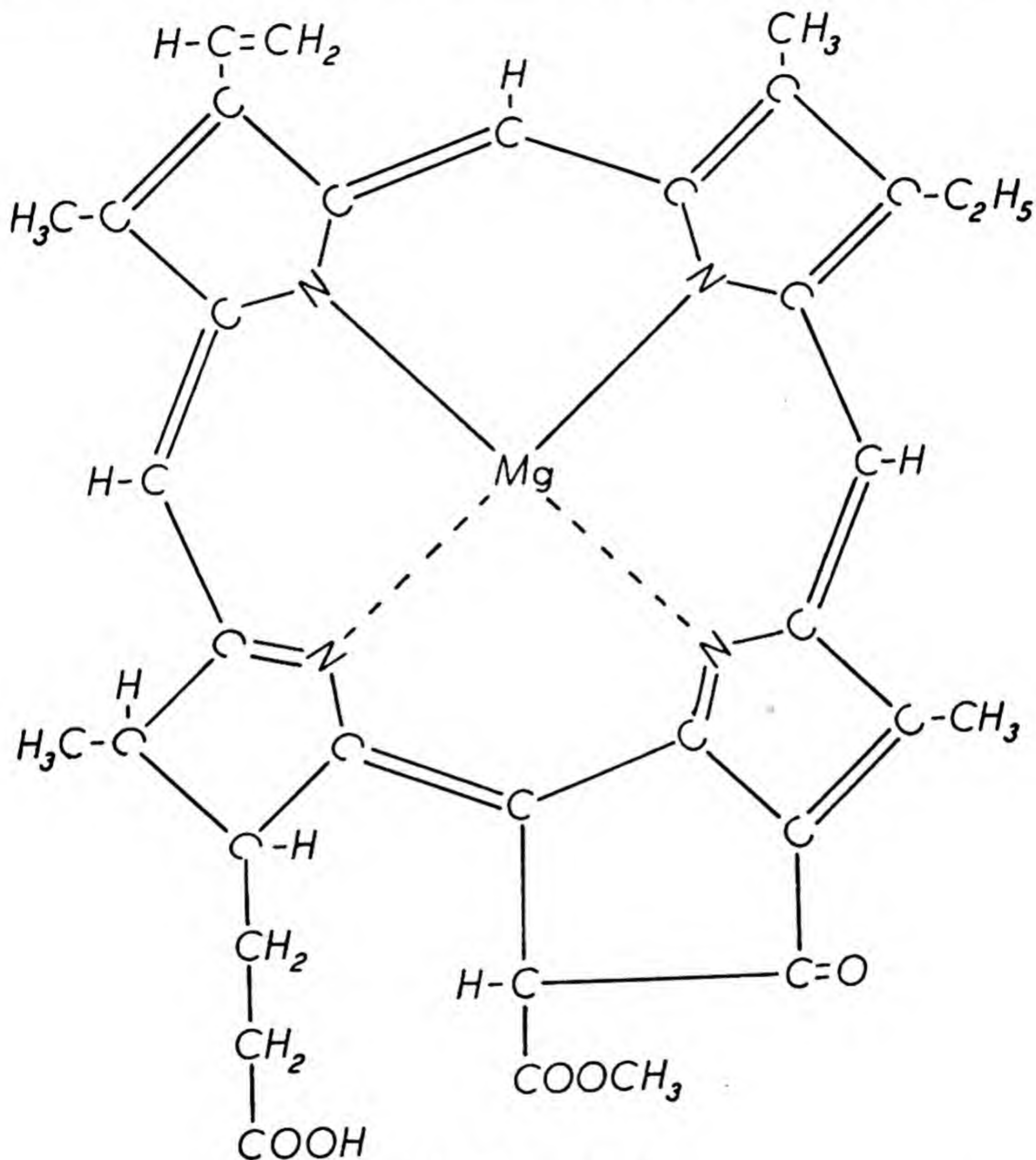
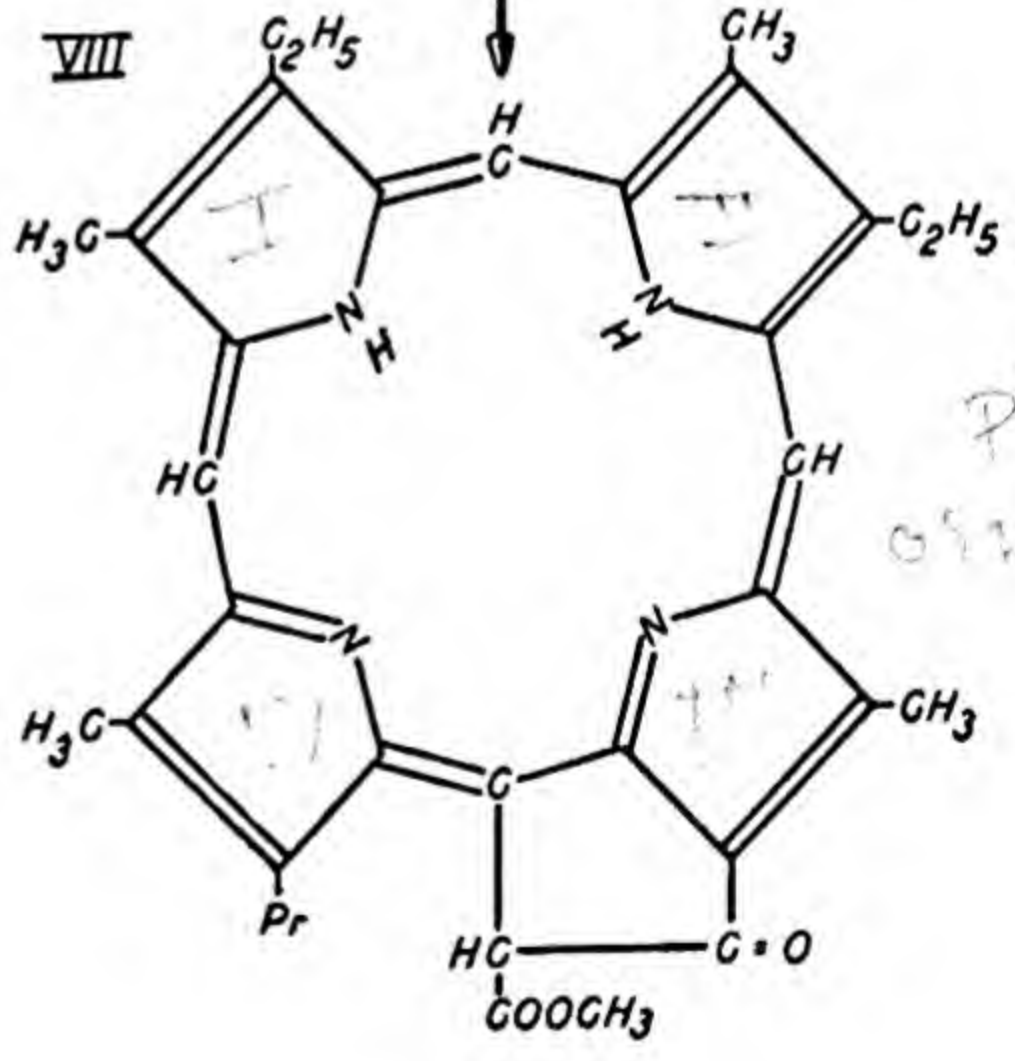
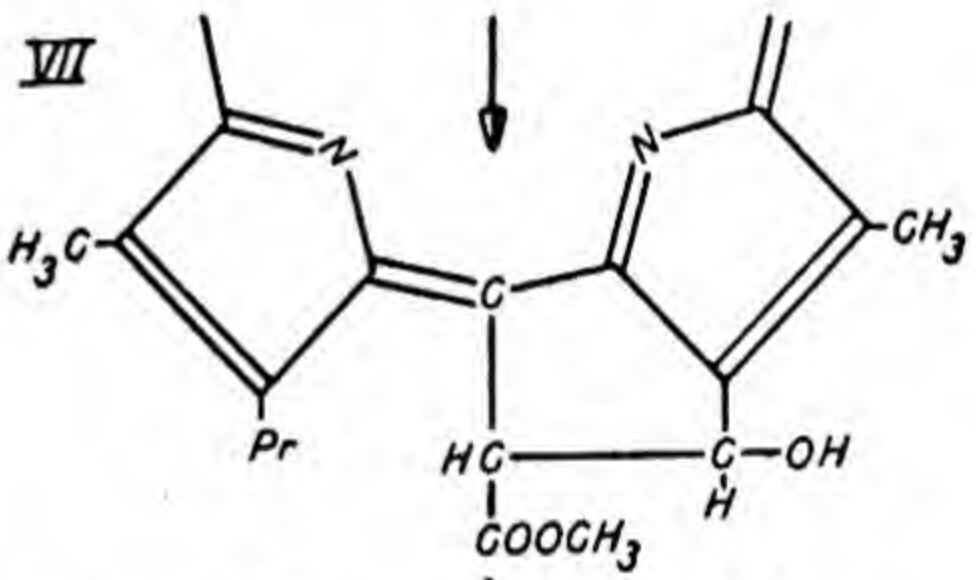
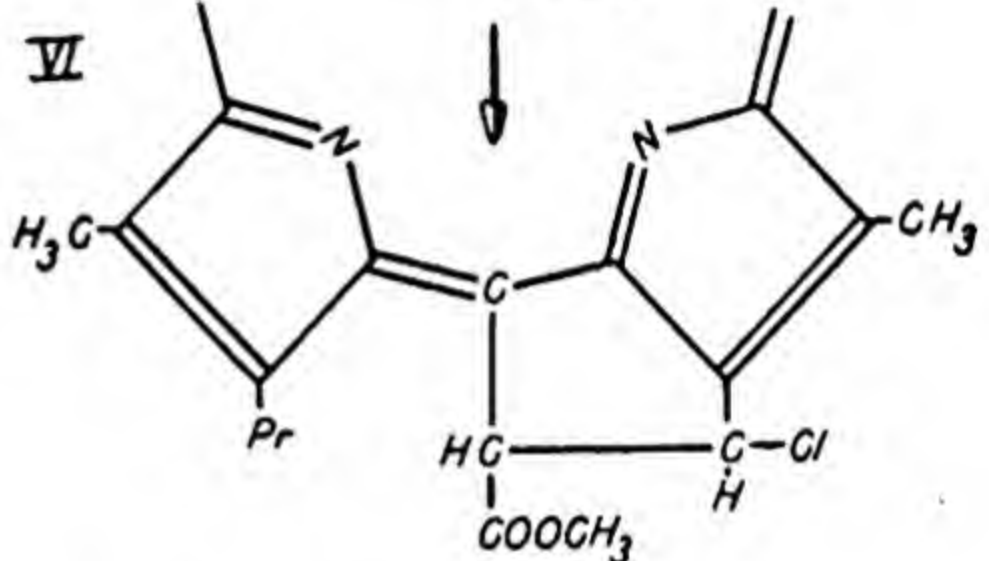
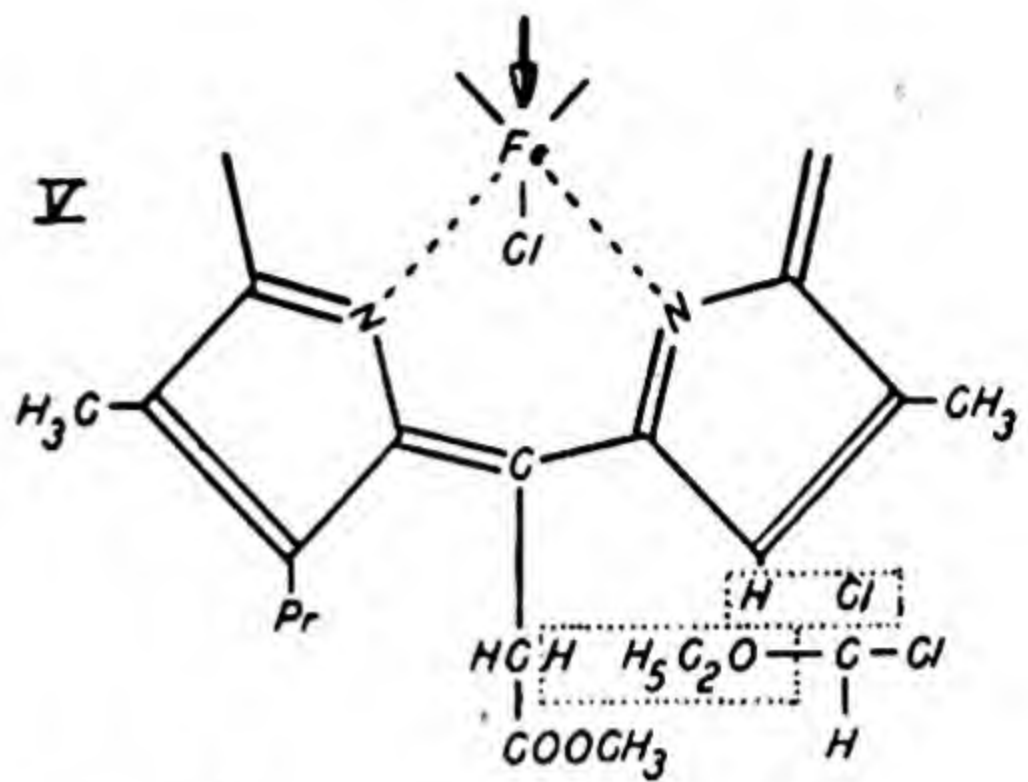
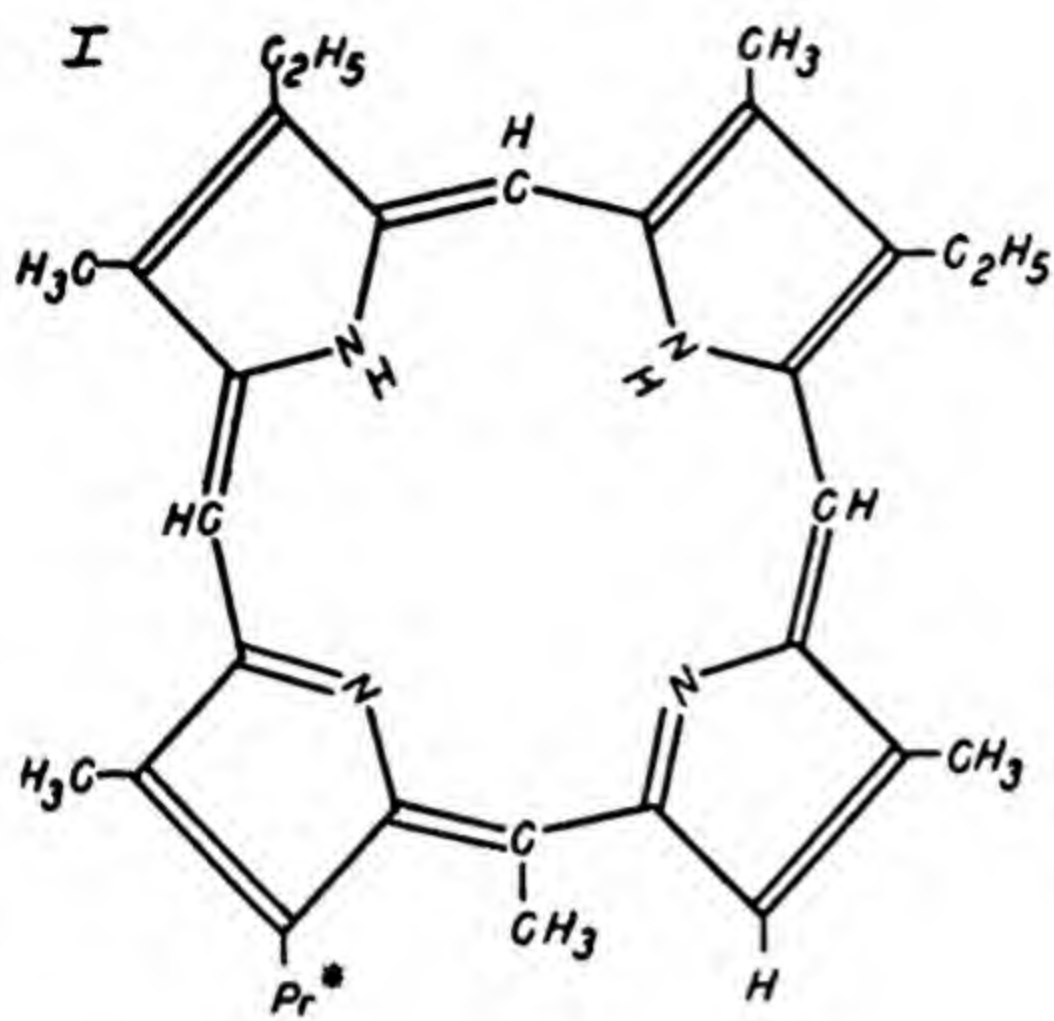


FIG. 8.7—Chlorophyllide *a*.

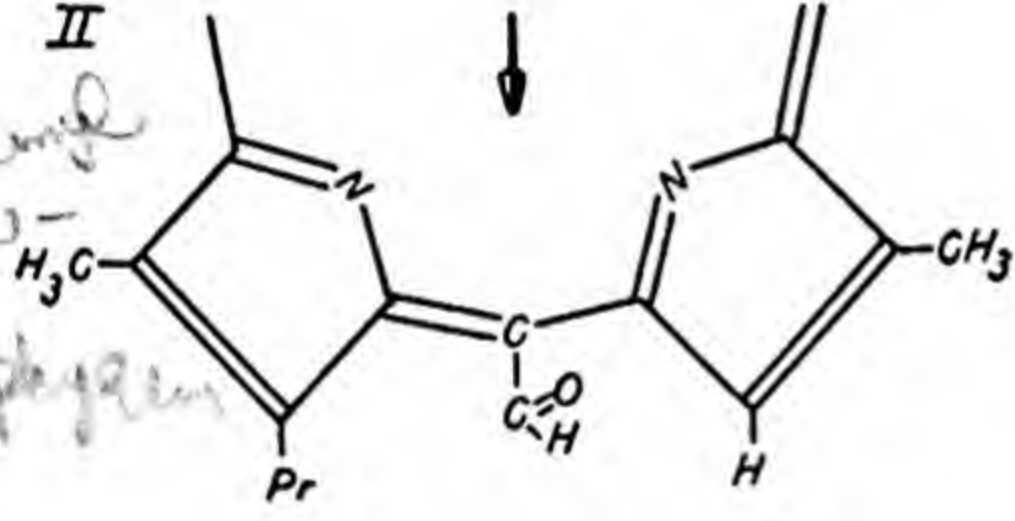
allowed to interact with dichloromethyl ether, the carbocyclic ring was closed (VI) and at the same time exchange of a chlorine atom for the hydroxyl group took place. The resulting 9-hydroxy-desoxopheoporphyrin (VII) was oxidized with CrO_3 to pheoporphyrin a_5 (VIII).

Fischer's synthesis of pheoporphyrin a_5 as sketched is of great

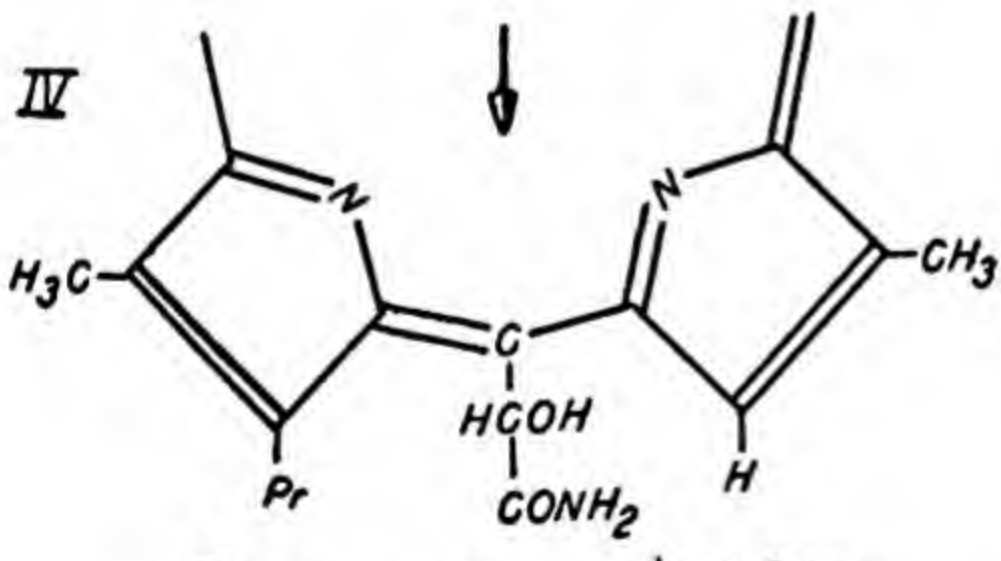
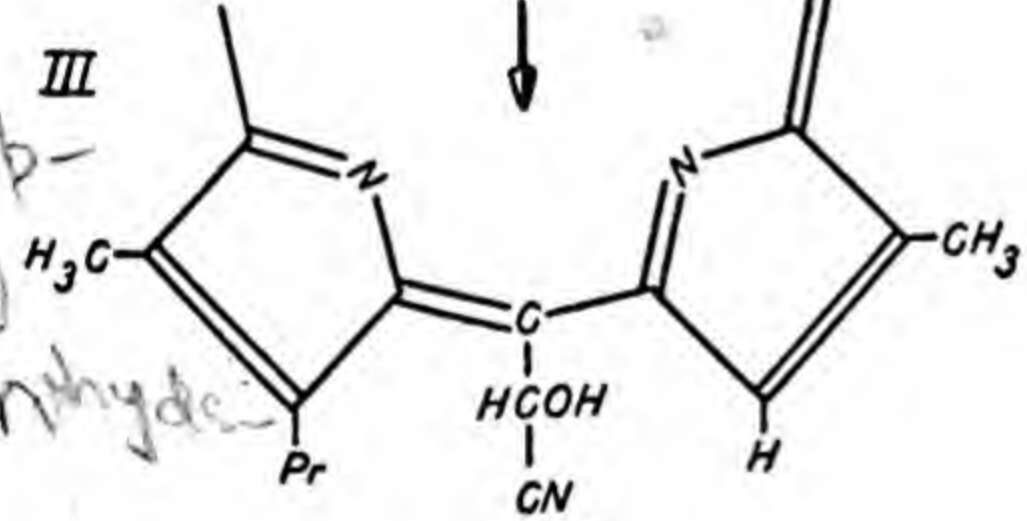
Phylloporphyrin



*γ-formyl
pyrro-
porphyrin*



*Corresp-
onding
cyanophyllide*



*Pyrroporphyrin
γ-glycolic acid
amide*

**Pr = -CH₂CH₂COOH*

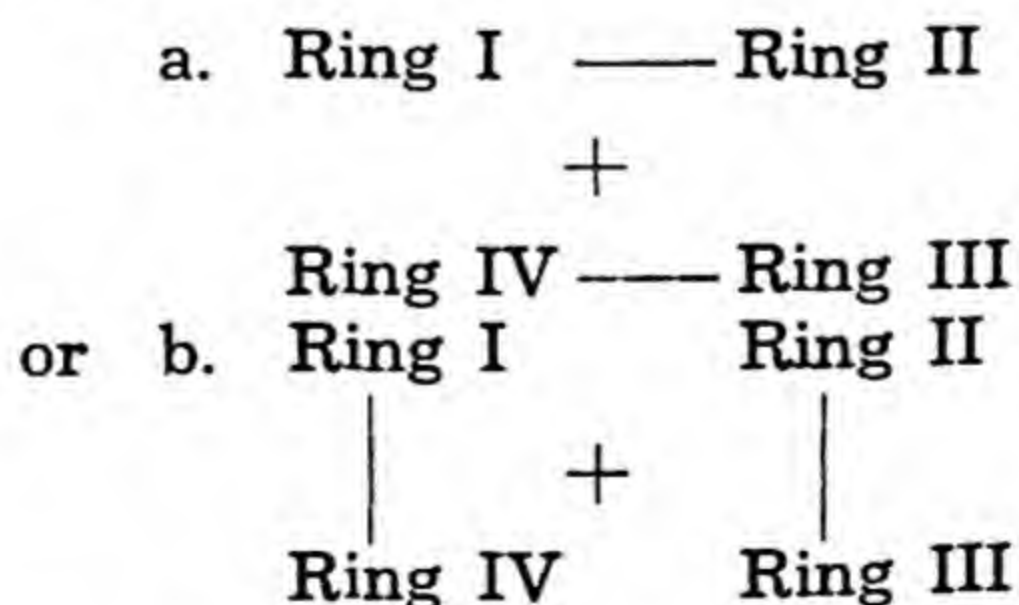
FIG. 8.8—Pheoporphyrin a.

importance as an approach to the ultimate synthesis of chlorophyll and as at least one route of adding the substituted carbocyclic ring to the porphyrin ring. It is relatively easy to remove the two hydrogen atoms in positions 7 and 8 in pheophorbide and to hydrogenate the vinyl group in 2 position to ethyl. The performance of this reaction in the opposite direction is not possible.

A method for the introduction of a vinyl into a porphyrin is available (7) and several synthetic hydrogenated porphyrins of as yet unknown structure have been reported in the literature. With the large amount of information concerning the chemistry of pyrrole on hand (8) the most important problem is at present the synthesis of 7,8-dihydro compounds of the phorbin type in which the 6, γ -ethanone configuration is suitably substituted.

One of the research projects in progress at the Charles F. Kettering Foundation is the systematic investigation of the scope of the porphyrin synthesis from pyrrole and aldehydes mentioned above (5). The porphyrins obtained, their derivatives are used in model experiments for the study of properties and behavior of the more complicated chlorophylls and their derivatives. Especially $\alpha,\beta,\gamma,\delta$ -tetraphenylporphine and its metal complex salts have through this work become easily available in relatively large quantities, and their study has given a large amount of information (9).

For the preparation of the dihydrogenated porphine ring system the controlled reduction of porphyrins, e.g., with hydrogen in the presence of a suitable catalyst, may be used. But the exact location of the place of hydrogenation in the molecule is difficult to ascertain. In order to synthesize phorbin with the hydrogenated ring IV (see Fig. 8.4) it becomes necessary to produce substituted dipyrrolyl methenes or "pyrromethenes" (Fig. 8.9, I) and pyrrolyl pyrrolenyl methenes (Fig. 8.9, II) and to find the conditions for their condensation. These compounds with two rings could be linked together either by method



A considerable number of intermediate products with modified pyrrole rings must be rendered available in quantity for this work. The preparation of some of these substances was, therefore, undertaken.

With A. F. Johnson the synthesis of 2,4-dimethyl-3,5-dicarbethoxy pyrrole (= "Knorr Pyrrole," Fig. 8.9, III) was simplified and applied to the synthesis of 2,4-dimethyl-3-acetyl-5-carbethoxy pyrrole (Fig. 8.9, IV) and its isomer, the 2,4-dimethyl-3-carbethoxy-5-acetyl pyrrole (Fig. 8.9, V); the yields increased in both cases from 50 per cent as reported in the literature to about 70 per cent,

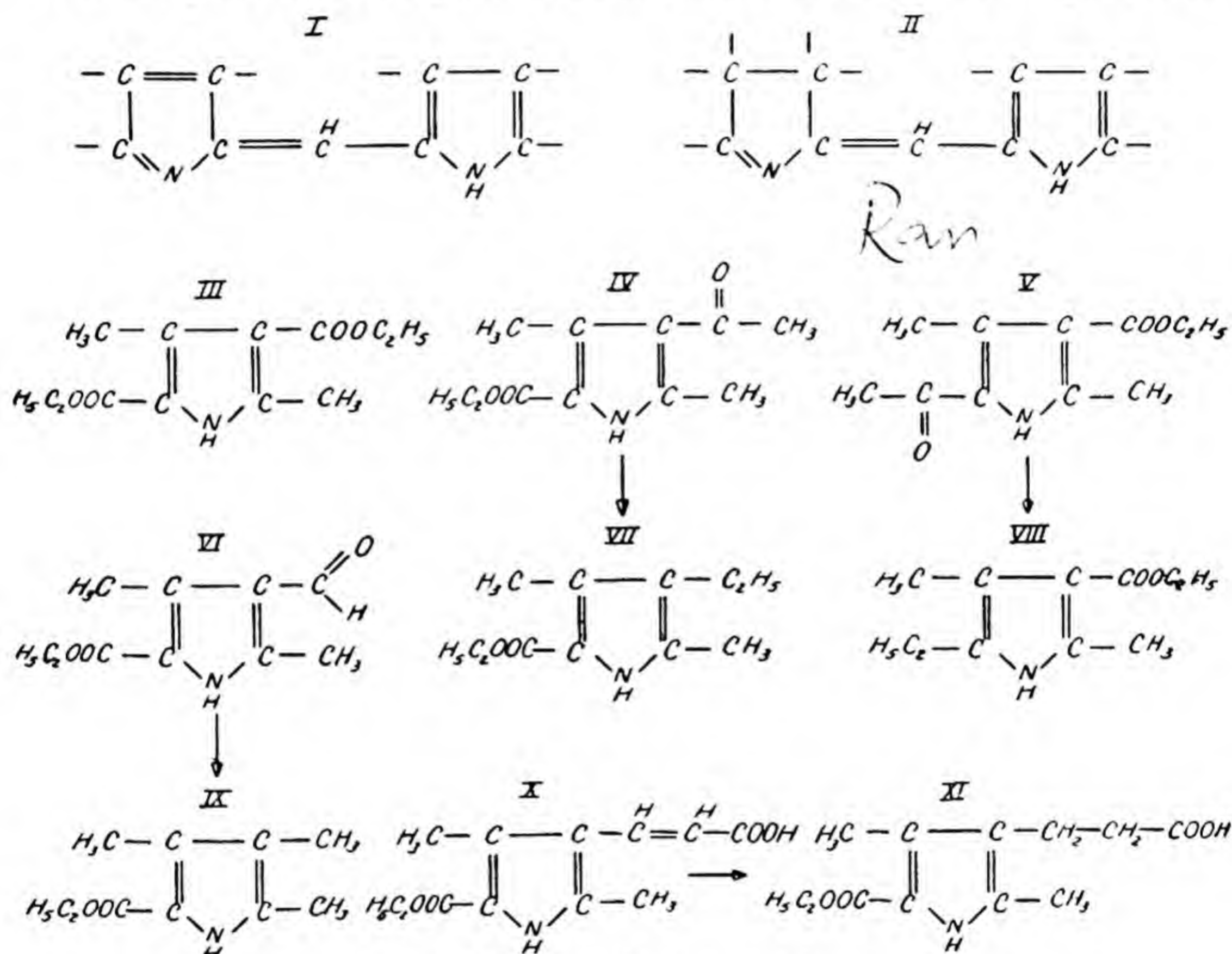


FIG. 8.9—Pyrrole compounds.

and the purity of the resulting materials was very high. Reduction according to Clemmensen of these two acetylated pyrroles gave very satisfactory yields of carbethoxylated cryptopyrrole (Fig. 8.9, VII) and its isomer VIII. This reduction method was also carried out on 2,4-dimethyl-3-formyl-5-carbethoxy pyrrole (= "Weiss Aldehyde," Fig. 8.9, VI) and gave 2,3,4-trimethyl-5-carbethoxypyrrole (Fig. 8.9, IX) in 76 per cent yield. 2,4-Dimethyl-3-acrylic acid-5-carbethoxy pyrrole (Fig. 8.9, X) was reduced to the corresponding propionic acid (Fig. 8.9, XI) with hydrogen at about 50 lbs. pressure with Raney nickel catalyst. The same catalytic method applied to 2,4-dimethyl-3-acetyl-5-carbethoxy pyrrole (Fig. 8.9, IV) yielded the carbethoxylated cryptopyrrole (Fig. 8.9, VII) in very pure condition and in 94 per cent yield directly. This was a considerable improvement over the method of preparing the free

cryptopyrrole first in an autoclave and then introducing the carbethoxy group. The experimental details on these methods are part of the dissertation presented by Arthur F. Johnson to the Graduate School of The Ohio State University in March, 1948, and will be published elsewhere.

The synthesis of suitably substituted pyrrolenes by synthesis or from pyrroles by chemical, catalytic, or electrolytic reduction has thus far always led to mixtures of hydrogenated reaction products of dihydro and tetrahydro type. The separation of the individual compounds in these mixtures offers great experimental difficulties, and the dihydrogenated material is always present in much smaller amounts than the completely hydrogenated pyrroles which are not fit for the purpose of synthesizing ring IV. This difficulty has rendered it impossible to apply the standard synthetic methods for dipyrrolyl methenes to the preparation of mixed methenes of the pyrrolyl pyrrolenyl type. Several possibilities to obtain this mixed type by direct synthesis are under investigation at this time.

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9.

Processes Accompanying Chlorophyll Formation

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WHEN grown in the dark, seedlings of most of the higher plants are yellow and contain no chlorophyll; instead, they contain traces of a green pigment called protochlorophyll. When these seedlings are illuminated under proper conditions of light intensity, temperature, and atmospheric content of oxygen, the leaves become green. This greening becomes visible after a very few hours and the formation of chlorophyll is clearly detectable in extracts of leaves after a few minutes. At room temperature the greening progresses at a rapid pace for several hours, but ultimately reaches what is apparently a limit (10a) .

Besides the formation of chlorophyll, many other chemical changes take place in the etiolated leaf during illumination. An examination of these changes may throw considerable light on the formation of chlorophyll and on the photosynthetic process itself.

Both photochemical and thermochemical reactions appear to be operative in some kind of integrated fashion during greening. It might be expected, therefore, that certain of these reactions could be disentangled from others by following the changes at different temperatures. Already some progress has been made by use of this procedure.

Temperature affects greening drastically. At high temperatures and at low temperatures, greening is greatly inhibited, perhaps completely inhibited (7, 9). When etiolated barley leaves are illuminated at 0°C., the leaves never exhibit a green color. This effect of temperature is a local effect, for if a leaf is illuminated under conditions so that one part of the leaf is maintained at 0°–4°C., and another part of the leaf is maintained at about 10°–12°C., that part of the leaf kept at the lower temperature remains yellow, whereas that part kept at the higher temperature greens normally.

Because the green color produced during illumination is due to chlorophyll, and because chlorophyll contains magnesium and is soluble in ether, one of the most obvious changes to follow during greening is the formation and transformation of the ether-soluble organic compounds of magnesium. For the purpose of following the changes in these magnesium compounds, etiolated barley seedlings were illuminated at different temperatures, and the leaves after being cut into short lengths were treated alternately with 80 per cent acetone and ether. The substances extracted were partitioned between ether and water. The magnesium content of the ether solution was determined and was designated "total ether-soluble magnesium." The chlorophyll in the ether solution was determined spectrophotometrically, and the quantity of magnesium contained in the chlorophyll—i.e., "chlorophyll magnesium"—computed. The changes brought about in the quantities of these two fractions, namely, the "total ether-soluble magnesium" and the "chlorophyll magnesium," by illumination of barley seedlings at different temperatures are shown in Figures 9.1 to 9.3.

In Figure 9.1, the changes in these two quantities are shown for different periods of illumination at room temperature, about 19°C. It is clear that initially the barley leaves contained a small quantity of magnesium compounds soluble in ether but that the quantity of chlorophyll was negligible. Illumination of the seedlings for two hours caused an increase in the total ether-soluble magnesium which was greater than the increase in the chlorophyll magnesium. (The source of illumination was a 100-watt Mazda lamp hung 40 cm. above the flat containing the seedlings.) Illumination for the next forty-six hours caused greater amounts of total ether-soluble magnesium to be produced, the increase of which was almost completely accounted for by the increase in chlorophyll magnesium. During the next twenty-three hours of illumination, there was no significant increase in either of these quantities. Whether the decrease noted in chlorophyll magnesium is real and significant is uncertain. Several results worthy of note were obtained from this experiment: The quantity of magnesium incorporated into the chlorophyll finally formed was far greater than the quantity of ether-soluble magnesium initially present in the leaves. Therefore, during the illumination, magnesium was incorporated into chlorophyll from inorganic sources. There was an acceleration of the rate of formation of organic compounds containing magnesium, especially chlorophyll, after the first two hours of illumination. The increase in total ether-soluble magnesium in the first two hours was greater than the increase in chlorophyll magnesium, but there-

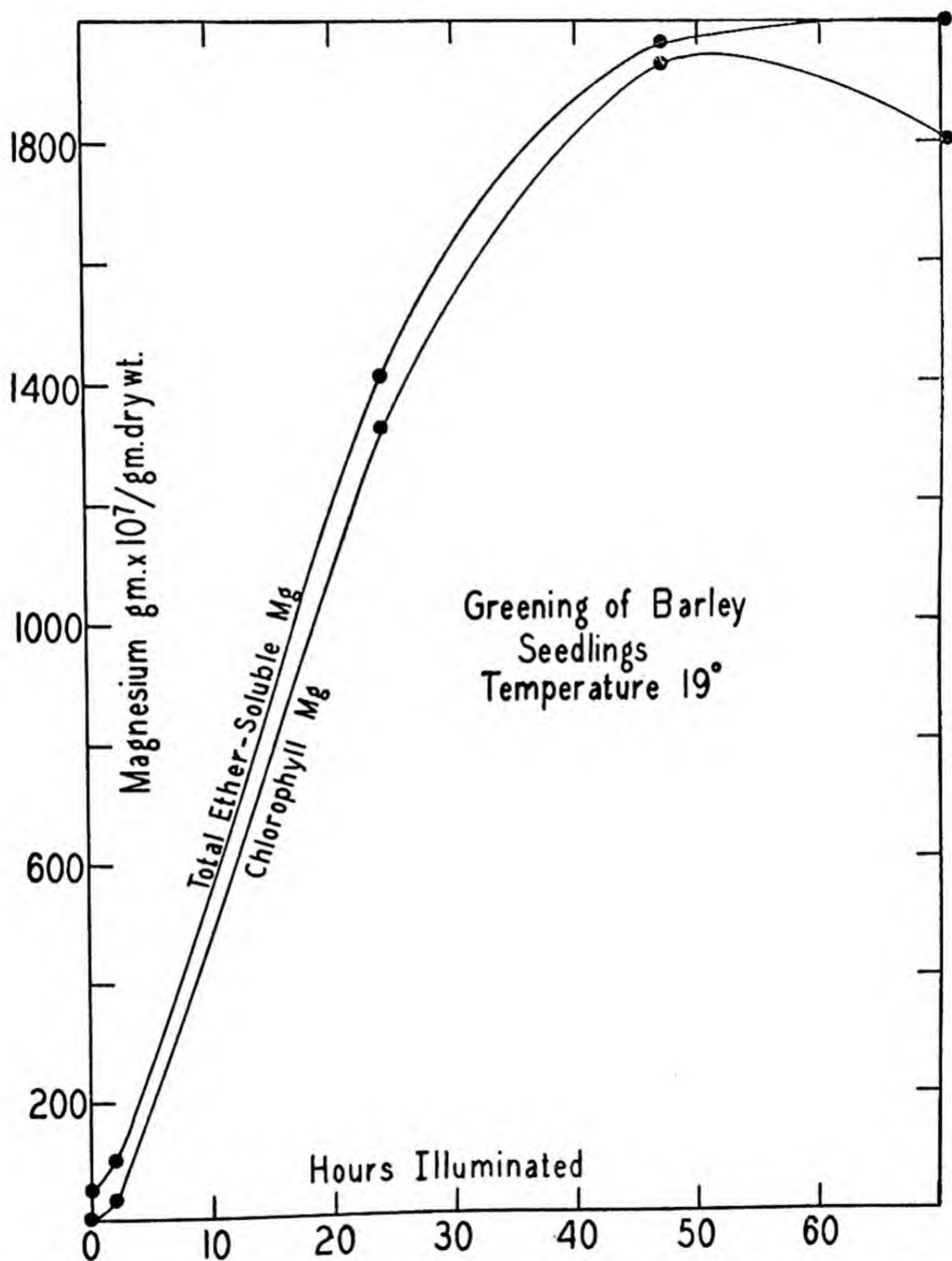


FIG. 9.1—Changes brought about in the quantities of total ether-soluble magnesium and chlorophyll magnesium by illumination of barley seedlings at 19°C. (Cf. 10a.)

after the increases in these two fractions nearly coincided. And the total ether-soluble magnesium and chlorophyll magnesium reached a limit.

The results obtained at 0°C. are shown in Figure 9.2. From these observations the following conclusions may be drawn: An initial amount of ether-soluble magnesium was present in the etiolated leaves which was far in excess of the magnesium that

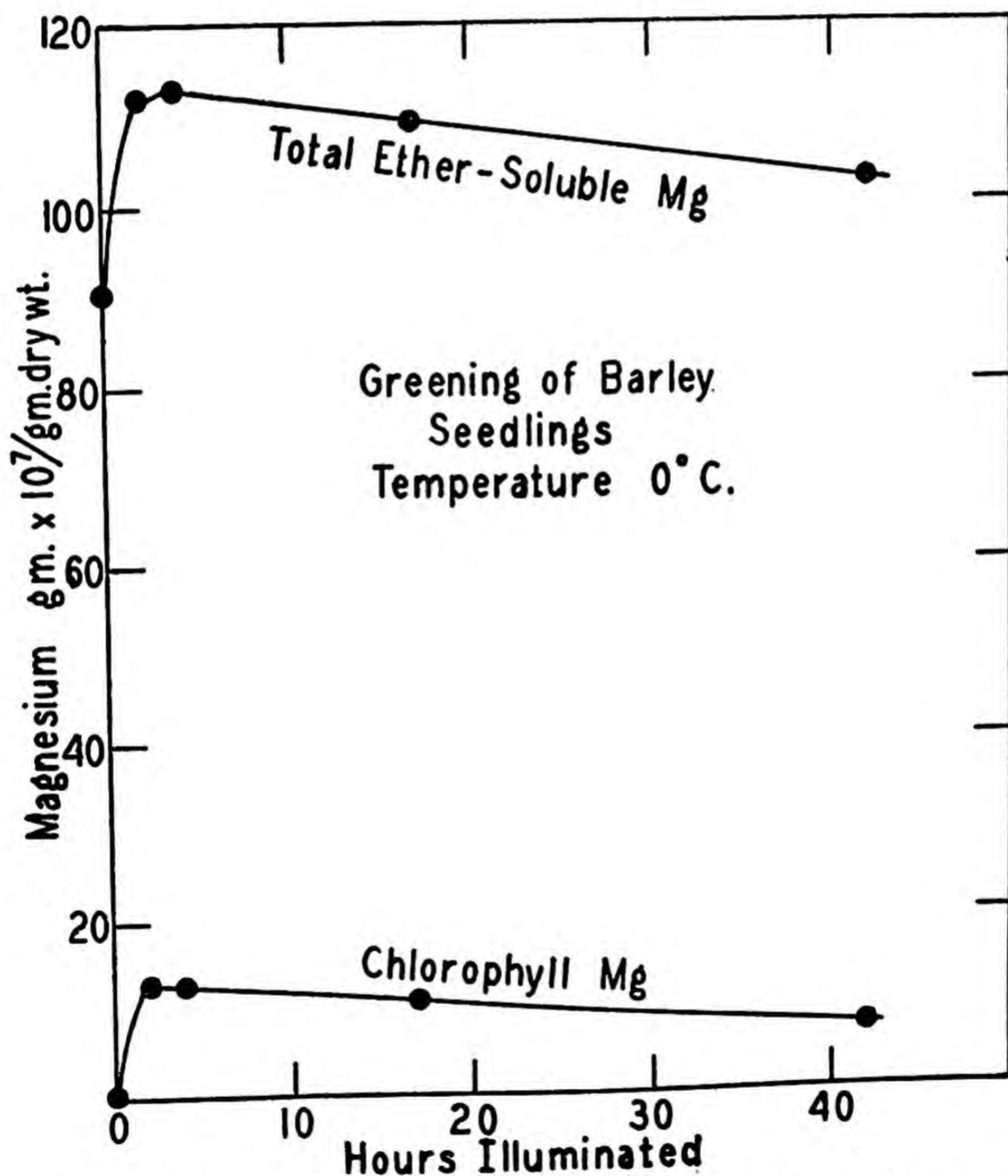


FIG. 9.2—Changes brought about in quantities of total ether-soluble magnesium and chlorophyll magnesium by illumination of barley seedlings at 0°C.

could possibly be attributed to chlorophyll. Only a small quantity of chlorophyll was formed, and that rapidly, upon illumination of the seedlings. The total ether-soluble magnesium increased to a much greater extent during two hours of illumination than did the chlorophyll magnesium. At about two hours' illumination both the total ether-soluble magnesium and the chlorophyll magnesium reached a maximum; thereafter both declined. On continued illumination the decrease in total ether-soluble magnesium was much greater than the decrease in chlorophyll magnesium. There is indication, therefore, that when metabolism was decreased the magnesium compounds were destroyed by light faster than they were produced.

Figure 9.3 shows the results obtained at 7°C. The following observations should be remarked: The initial quantity of ether-soluble magnesium was considerably greater than could be accounted for by any chlorophyll which was present. Both total ether-soluble magnesium and chlorophyll magnesium increased during the first two hours of illumination and to nearly the same extent. This was true up to seventeen hours, but at forty-three hours the increase in total ether-soluble magnesium considerably exceeded the chlorophyll magnesium. In the curves of Figure 9.3, three distinct stages are discernible in the process of greening: a rapid increase in the ether-soluble compounds containing magnesium which was initiated by illumination—a photochemical transformation; a period of several hours in which the rate of increase was relatively small; and a period of accelerated production of the magnesium compounds concerned.

The curve at 7°C. appears to combine the effects shown in the curves for 0° and 19°C. It shows that there is an initial photochemical reaction. This presumably acts as a trigger reaction for supplying materials which are formed into precursors of chlorophyll. These precursors, on further illumination, are transformed through photochemical and thermochemical action into chlorophyll at an accelerated pace. The precursors formed are probably organic compounds containing magnesium, as the large increase in total ether-soluble magnesium relative to chlorophyll magnesium indicates.

The protochlorophyll present in etiolated leaves contains magnesium. Until the present time, it has been impossible to determine how much of the magnesium initially present in etiolated leaves is due to this pigment. Recently, protochlorophyll has been isolated from etiolated barley seedlings and its magnesium content and specific absorption coefficients at different wave lengths of light determined (6). From these measurements it has been possible to

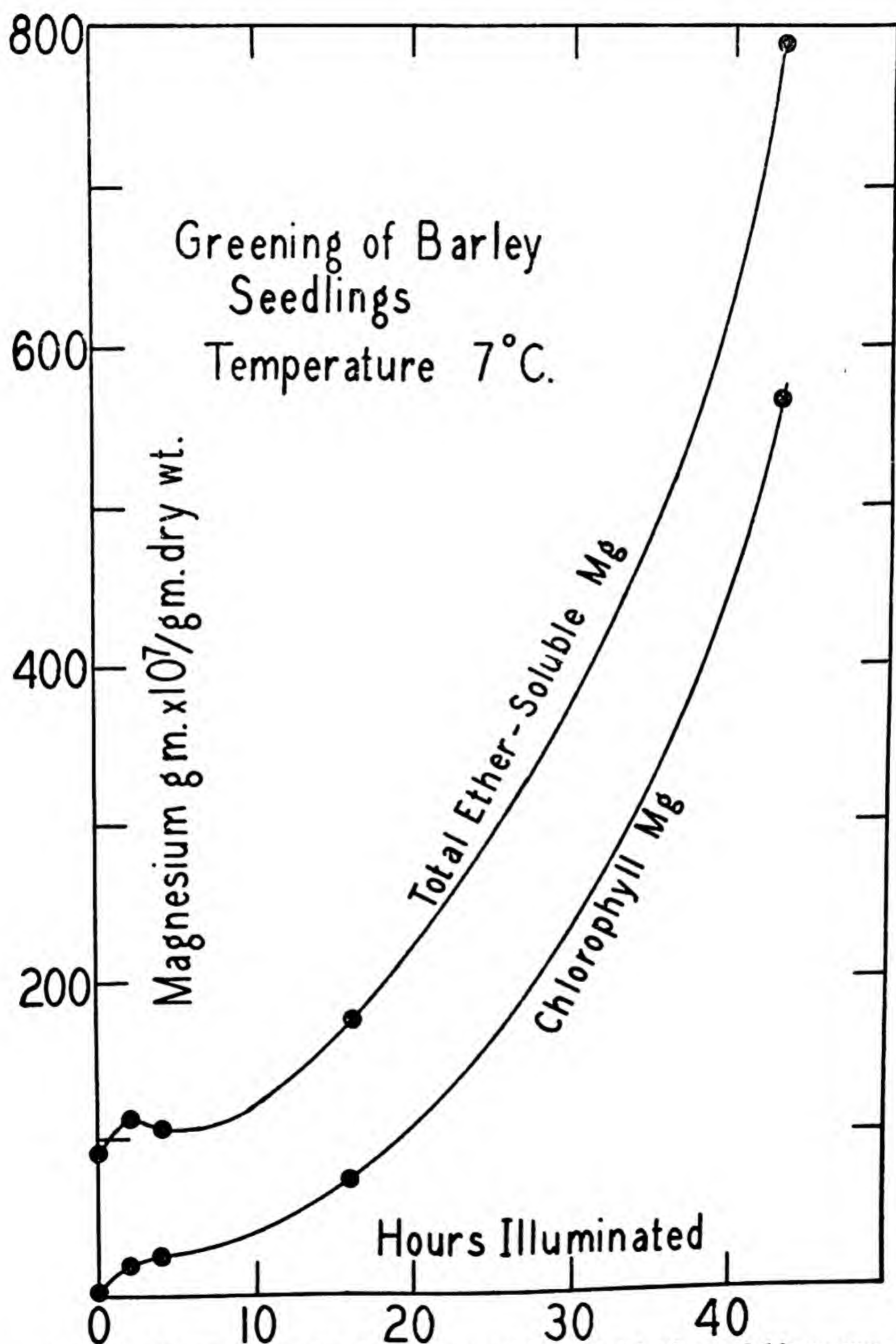


FIG. 9.3—Changes brought about in quantities of total ether-soluble magnesium and chlorophyll magnesium by illumination of barley seedlings at 7°C.

calculate what proportion of the total ether-soluble magnesium obtained from etiolated barley leaves is accounted for by protochlorophyll. The calculations show that the proportion differs in different samples of leaves and varies from about 23 to 46 per cent.

During the initial period of illumination, the protochlorophyll disappears and chlorophyll appears. Largely on the basis of this observation, it has been assumed that protochlorophyll is converted to chlorophyll by photochemical action (for review of this subject cf. [5]). But on the strength of this fact alone, such an assumption is scarcely valid. It is conceivable that the disappearance of protochlorophyll and appearance of chlorophyll are independent of each other.

Recent observations, however, have given additional evidence for the conversion of protochlorophyll to chlorophyll. At 0°C. the quantity of chlorophyll formed reaches a maximum in something less than two hours (cf. Fig. 9.3). There appears to be a limited quantity of precursor which is transformed to chlorophyll by illumination and which is not regenerated at this low temperature. Experiments at low temperature, therefore, offer a means for making a quantitative correlation between the amount of this precursor and the chlorophyll formed. If protochlorophyll is the precursor, then the amount of chlorophyll formed should be proportional to the quantity of protochlorophyll initially present. This is the case (10b). Furthermore, quantitative data show that at low temperatures there is about a mole-for-mole correspondence between the amount of protochlorophyll which disappears and the amount of chlorophyll which is produced. These facts combined with the remarkable agreement of the absorption curve of protochlorophyll (6), and the action curve for the formation of chlorophyll (3) leave little room for doubt that in the initial stages of greening protochlorophyll is transformed to chlorophyll.

Further greening is, however, contingent on the formation of more precursor, and this appears to be an integration of photochemical and thermochemical reactions, as the results at 7°C. indicate. Protochlorophyll is regenerated in the dark (8), but whether this is the thermochemical reaction responsible for the further production of chlorophyll remains to be determined from physiological experiments now under way.

In etiolated barley seedlings, the transformation of protochlorophyll to chlorophyll at low temperatures forms only chlorophyll *a* (cf. 4). Just when chlorophyll *b* appears, and under what conditions, is still a question. It may be significant that we have been unable to demonstrate the presence of any protochlorophyll *b* in the seedlings. The transformation of protochlorophyll to chlorophyll

is carried on in the plant tissue, but so far has not been observed to occur in organic solvent extracts of plant tissue.

On the basis of the structural formulas accepted at the present time for protochlorophyll (1) and for chlorophyll (2), the transformation of protochlorophyll to chlorophyll is a reduction. It is

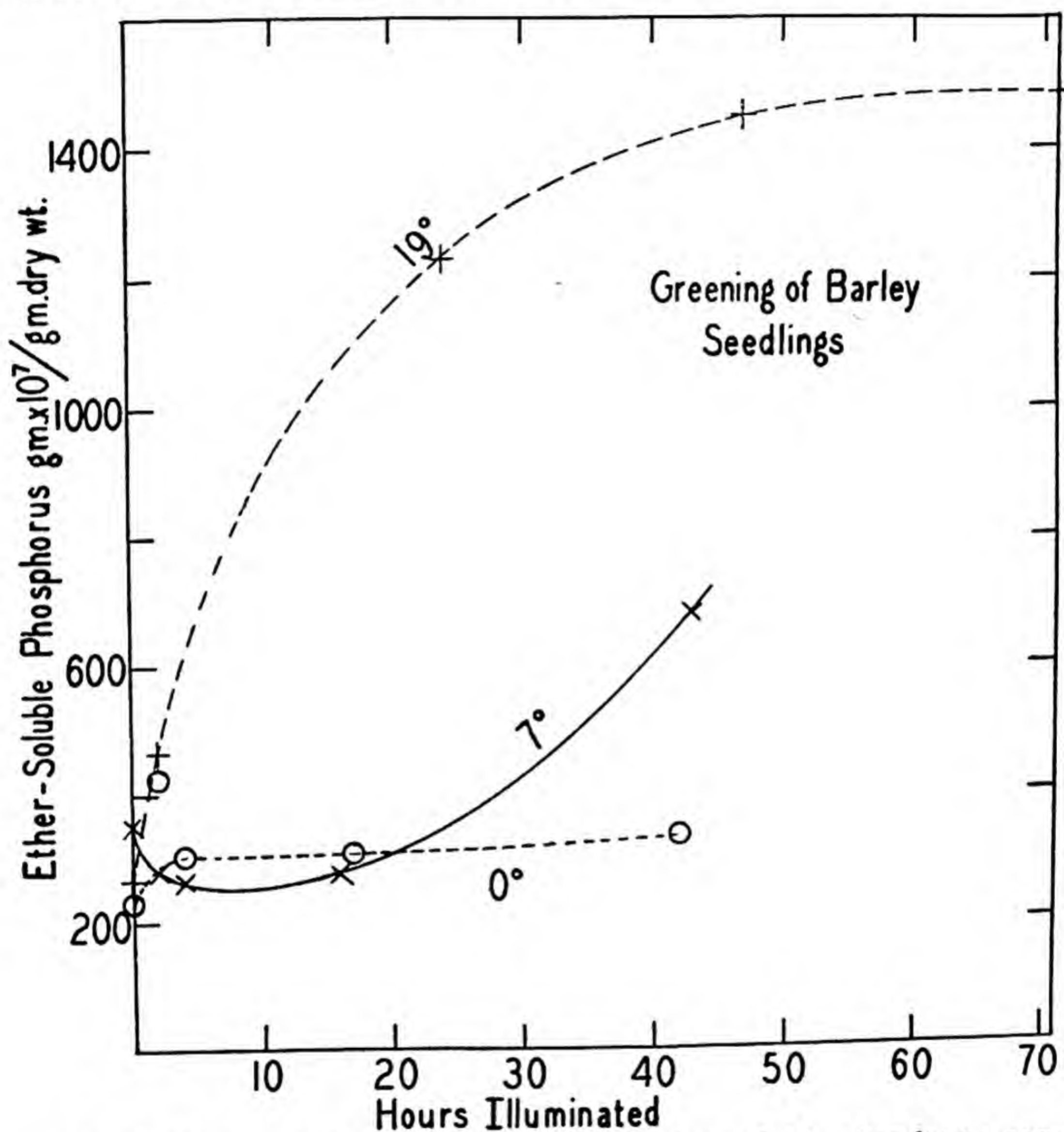


FIG. 9.4—Comparison of the quantities of ether-soluble phosphorus compounds formed at 0°, 7°, and 19°C. during illumination of etiolated barley seedlings.

suggestive that this transformation, so closely allied with the photosynthetic process, the reduction *par excellence*, is itself a reduction.

During the greening of etiolated barley seedlings at room temperature, ether-soluble phosphorus compounds increase at a rapid rate (10a). This change may be of importance in photosynthesis inasmuch as the transformation of carbohydrates is known in many

instances to depend upon the formation of phosphorus derivatives.

The question naturally arose whether the incorporation of phosphorus into organic compounds is a photochemical or a thermochemical reaction or a combination of the two. Comparison of the action at 0°, 7°, and 19°C., as shown in Figure 9.4, strongly indicates that the action is thermochemical but closely allied with the formation of chlorophyll. At 7°C. there was a relatively long inhibition period before the rapid rise of organic compounds of phosphorus began, reminiscent of the behavior of chlorophyll. At 0°C., the ether-soluble phosphorus showed no significant gain. These facts suggest that the formation of ether-soluble phosphorus compounds is not primarily a photochemical action but depends on the formation of chlorophyll and its action.

Other processes are initiated during the formation of chlorophyll. Chief among these is the ability to carry on photosynthesis. While there seems to be some difference of opinion concerning the details of the development of this action in its relationship to chlorophyll formation, the fact remains that in the higher plants no photosynthesis is known to take place without chlorophyll.

In conclusion, it can be said that greening is a very complicated process in which both photochemical and thermochemical reactions play a part. The photochemical transformations appear to affect particularly the magnesium compounds of the etiolated leaf, one such reaction being the formation of chlorophyll from protochlorophyll. These reactions are accompanied by other changes in the leaf, such as the formation of organic compounds of phosphorus and the genesis of photosynthetic capacity. The correlation of the processes accompanying the formation of chlorophyll may aid in the clarification of our concepts concerning the mechanism of the photosynthetic process.

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10.

The Quantum Requirement of Photosynthesis

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University of Illinois

CONFLICTING values have been reported for the quantum requirement of photosynthesis. The finding of Warburg and Negelein (14, 15) that a minimum of four absorbed quanta suffices per molecule of O_2 produced, has not been confirmed by American investigators (1, 5, 6, 8, 11), whose results indicate that a value of about 10 is the minimum thus far obtainable. On the other hand, Eichhoff (4), and Ornstein and co-workers (10) found values approximating the yield reported by Warburg and Negelein.

Only in the investigations of Rieke, and of Emerson and Lewis, was the technique so closely similar to Warburg's that detailed comparison is possible. They, like Warburg and Negelein, used the green alga *Chlorella pyrenoidosa*, and measured photosynthesis by means of a differential manometer and cathetometer. If they also computed their rates of photosynthesis in the same way as Warburg and Negelein, the results indicated a quantum requirement of about four. Indeed, Emerson and Lewis found in some cases an apparent requirement of only three quanta per molecule of evolved O_2 . However, they, as well as Rieke, had noted that these high efficiencies were obtainable only when photosynthesis and respiration were measured in a sequence of short periods of light and darkness (fifteen minutes each), and that the highest yields depended on choice of certain arbitrary time intervals for computation of the net pressure change attributed to photosynthesis. Pressure measurements over longer periods of light and darkness, after steady rates had been established, led to larger quantum requirements, in the neighborhood of 10 per molecule of evolved O_2 .

Emerson and Lewis investigated the origin of the difference in yields as computed from the time intervals used by Warburg and Negelein, and as computed from longer time intervals, and found

that the change from darkness to illumination was followed by a sudden and temporary increase in rate of positive pressure change. Darkening produced a less marked but also temporary excessive rate of negative pressure change. These temporary deviations from steady rate were easily overlooked when pressure changes were read at five-minute intervals, and with usual speeds of shaking of the manometer vessels, as in the procedure of Warburg and Negelein. But when readings were taken at one-minute intervals, and with stronger shaking of the vessels, to give improved mixing of the cell suspension and more rapid distribution of physiologically exchanged gases between gas space and suspending fluid, the deviations became so prominent that it was impossible to overlook them.

Emerson and Lewis (6) reported on a detailed study of these deviations from steady rate of pressure change. Their experiments

showed that the ratio of exchange of O_2 and CO_2 $\left(\frac{CO_2}{O_2} = \gamma \right)$ was

not constant, as had been assumed by Warburg and Negelein in the computation of their results. Exposure to light was followed by a temporary deficit in CO_2 consumption (at higher light intensities, the deficit became a positive CO_2 production), and in a subsequent dark period there was a corresponding temporary deficit in CO_2 production, as compared to O_2 exchange. They noted that a certain combination of time intervals and conditions led to changes in γ which substantially increased the pressure change apparently attributable to photosynthesis. The same combination of intervals and conditions had been used by Warburg and Negelein for obtaining maximum quantum yields. Since, in their own experiments, quantum numbers of four or less were obtainable only by computation of photosynthesis on the clearly erroneous assumption of constancy of the value of γ , and since proper regard for the changes in γ led to a quantum requirement of about ten, they concluded that Warburg and Negelein's low quantum numbers were probably the result of similar fluctuations in γ , and were, therefore, to be rejected. Emerson and Lewis' interpretation found general credence among American investigators, because no one, by using various methods, had been able to achieve quantum numbers appreciably lower than ten, a minimum value which had been confirmed in several independent studies (cf. 7).

Warburg (13) reports that he has re-investigated the exchange ratio γ , and has failed to find the fluctuations reported by Emerson and Lewis. According to him, γ is essentially constant, even over short periods of light and darkness. He, therefore, rejects the inter-

pretation given by Emerson and Lewis to his earlier results, and on the basis of new measurements, confirms his original finding that a minimum of four absorbed quanta per molecule of evolved O_2 suffices for photosynthesis. He neglects the fact that Rieke, as well as Emerson and Lewis, reported close duplication of his numerical results, and attributes their lower efficiency values to various factors which are discussed below.

There is, therefore, complete disagreement between the conclusions of Warburg and those of Emerson and Lewis, and consequent uncertainty as to the quantum yield, in spite of close similarity of the methods used by these two groups of investigators. Two possible sources of disagreement may be considered. Either the biological material behaves differently in the two cases, so that the same method leads inevitably to different results, or there are differences in application of the method which lead to different results in spite of uniform behavior of the material.

Much evidence could be adduced against regarding the biological material as the source of the disagreement. However, because of the complexity and variability of living organisms, it would be difficult to exclude this possibility. Supposedly the alga *Chlorella pyrenoidosa* was used in both sets of investigations, but species of *Chlorella* are distinguished only with difficulty, and even if it were established that the same strain had been used in each case, there would remain the possibility of differences arising from conditions of growth or handling of the cultures. Those who have studied the physiology of algae in relation to culture conditions will realize that the only sure way of establishing comparability is to grow the cultures in the same laboratory under identical conditions. Every effort is being made to carry out such a comparison between the strains used by Warburg and by Emerson and Lewis. Whether or not this proves feasible, we must also consider the experimental technique as a potential source of differences.

Warburg's short-period γ measurements, as well as those of Emerson and Lewis, were made manometrically. Because of the difference in solubility of CO_2 and O_2 in water, the pressure change Δh caused by a given exchange of these two gases will depend upon the ratio of gas and liquid volumes of the system in which the exchange takes place. If duplicate measurements are made with two different ratios of gas and liquid volume, two values, H and H' are

obtained, and γ is a function of the ratio $\frac{H'}{H}$, (cf. 12, p. 108).

Strict comparability of H and H' is essential for determination of γ by this method. A comparison of the technique of Warburg and

of Emerson and Lewis shows that the latter made better provision for maintaining comparability. They used a fixed volume of cell suspension in vessels of unequal size, while Warburg used a single vessel volume with unequal volumes of cell suspension (cf. 6, Fig. 1; 13, Fig. 1). When a given gas exchange takes place under comparable conditions in two different volumes of cell suspension, there is a possibility that mixing of the smaller volume will be more rapid, and consequently that the response to a changing rate of gas exchange will manifest itself more rapidly. The values H and H' measured over the same time interval would then not be comparable until steady rates had been reached in both cases.

Warburg recognized this possibility, and made an arbitrary and in our opinion incorrect adjustment in the values of H and H' for the initial ten minutes of illumination. By making this adjustment he obtained a constant value of γ , instead of finding the fluctuations reported by Emerson and Lewis. In the case of their γ measurements, no adjustment was called for, since the same volume of cell suspension was used for measuring both H and H' , and mixing was assumed to be comparable. There may have been differences in the mixing of the two unequal gas spaces in their experiments, but since the gas was well mixed by the agitated liquid surface, differences in mixing of the two gas volumes were regarded as negligible.

Further doubt is cast upon the comparability of Warburg's values for H and H' by his procedure of measuring these two values in succession on a single sample of cells. Successive measurements on a single sample seldom give exactly the same results, in spite of careful control of the constancy of external conditions. The rate of respiration usually shows a progressive change during the course of a series of measurements, and at best the correction applied to the light readings from respiration measurements made before and after the light exposures is only an approximation. The closeness of this approximation appears to change somewhat over the course of an experiment. Therefore, Emerson and Lewis, who also made their determinations of H and H' in succession, used a fresh aliquot of the same stock of cell suspension for each set of measurements.

A third possible source of non-comparability of Warburg's values of H and H' is the difference in light absorption. As long as the vessels are at rest, the light absorption per unit area of the illuminated surface of the vessel will be the same, regardless of the depth of cell suspension. But when the vessels are shaken, the smaller volume will be subject to greater changes in depth as the fluid washes from end to end of the vessel. Unless there are enough cells to insure total absorption with both the fluid volumes used, the light absorption will vary with the fluid volume, making H and H' not

comparable. Emerson and Lewis avoided this source of error by using the same fluid volume for H and H' . Warburg sought to test for the equality of absorption in his two different fluid volumes, by interrupting the shaking for a five-minute interval during the illumination period. We repeated this test, under the conditions specified by Warburg, and found that the rest period of five minutes, followed by five minutes of shaking, is too short to give conclusive evidence of equality of absorption. Visual inspection shows that under the conditions specified by Warburg, the shaking results in changes in depth too great to maintain comparable absorption, in spite of the fact that a five-minute interval without shaking may indicate no appreciable loss of light absorption.

In order to see how much error could be introduced by these sources of non-comparability of H and H' , we have repeated Warburg's measurements, following his specifications in detail. We found that errors due to making successive sets of measurements on a single sample, and to differences in light absorption for the large and small fluid volumes, are small compared to the error arising from the arbitrary adjustment made by Warburg to compensate for supposed differences in rate of response to changes in gas exchange for the different fillings of the vessel. Since the errors introduced in this way are sufficient to account for the difference in conclusions reached by Warburg and by Emerson and Lewis, we shall neglect the other sources of error mentioned above, and give special attention to Warburg's adjustment of H and H' for differences in rate of diffusion.

In Figure 10.1 we have plotted Warburg's pressure measurements from his data (13, pp. 199-200). The vessel was first filled with 5 ml. of suspension, and the upper sequence of measurements was made. Then 3 ml. more of liquid were added, and the lower sequence was made. The figure shows that for the 5 ml. volume, the changes from dark to light and from light to dark resulted in almost immediate attainment of the new rate of pressure change, while for the 8 ml. volume there was an apparent delay in response following both the dark-light and light-dark transitions. Warburg attributes this delay to slower diffusion in the 8 ml. volume. Therefore, in computing the values of H and H' attributable to photosynthesis during the first five minutes of light, he adds what he calls "after-effect," namely the apparent deficit of pressure decrease for the first five minutes dark, to the pressure increase observed during the first five minutes light. This adjustment makes a substantial increase in the value of H' (8 ml. volume) for the first 10 minutes of light. The effect of this increase is to make the value of H' nearly constant throughout the light exposure. Corresponding adjustment in the value of H is so small that it is negligible, and even without

adjustment, H would be nearly constant throughout the light period. Therefore, the large adjustment in H' is responsible for making the ratio H'/H constant for the light period. Constancy of H'/H indicates constancy of γ , so Warburg concludes that there are no important changes in γ for the gas exchange due to illumination.

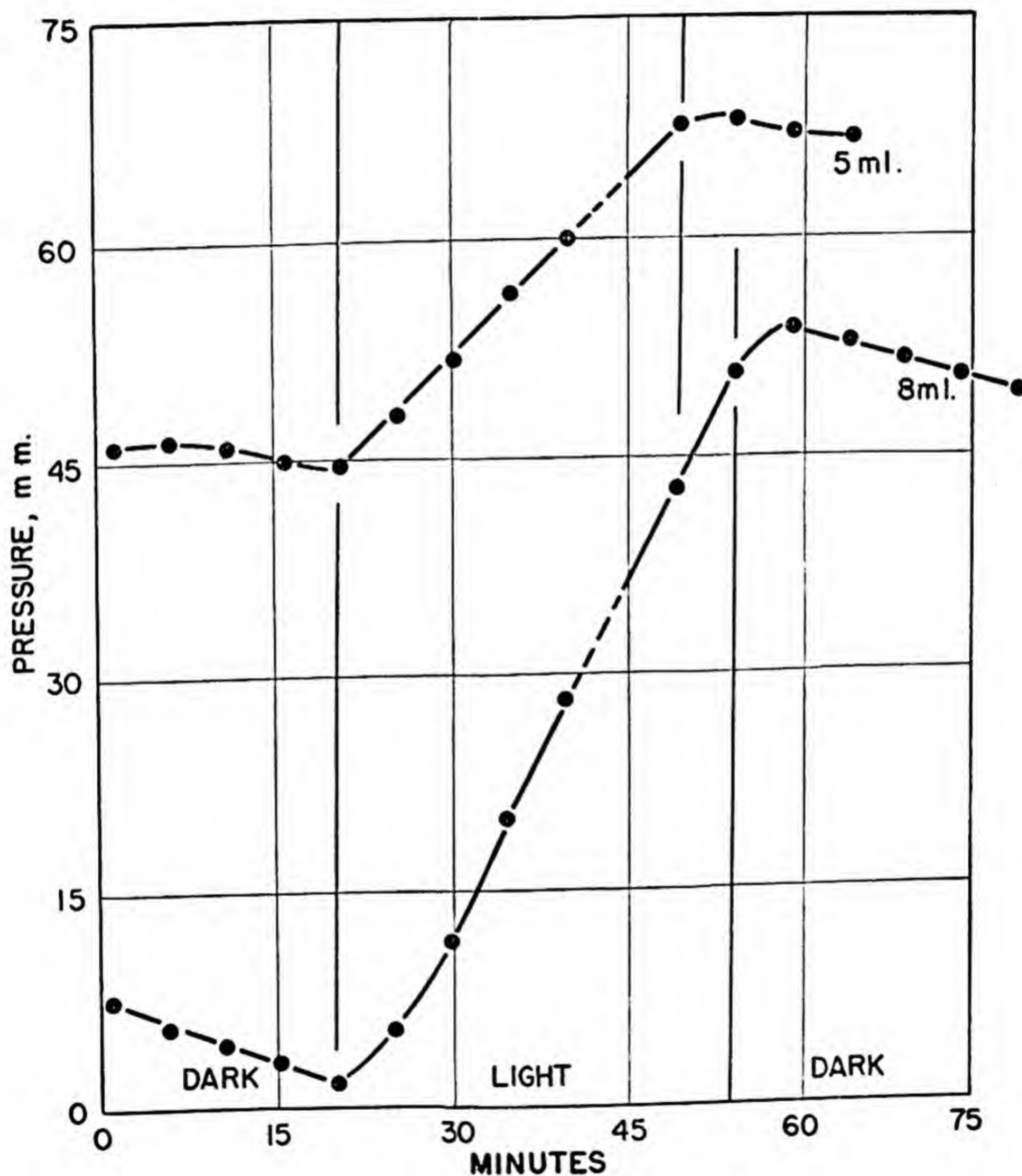


FIG. 10.1—Plot of data from Warburg's manometric γ determination. Conditions of experiment specified by Warburg: 20°C., 100 cmm. cells suspended in culture medium saturated with 10 per cent CO_2 in O_2 . Light from incandescent lamp filtered through red glass. Volume of vessel 14.47 ml. The readings with 5 ml. fluid volume were made first. Then 3 ml. culture medium were added, making a total fluid volume of 8 ml., and the sequence of readings repeated. The broken sections of curves indicate five-minute intervals when vessels were allowed to stand unshaken to test for totality of light absorption.

The computation of deficit of pressure change during the early part of the dark period, and the addition of this computed deficit to the pressure change observed during the early part of the preceding light period, implies the assumption that both the computed deficit and the observed pressure change represent the same ratio of gas exchange. In other words, the adjustments of H and H' imply the assumption that γ for the gas exchange due to light was the same at both the beginning and end of the light period. In our opinion, this makes the adjustments inadmissible, because the purpose of the experiment was to test the constancy of γ . If no adjustments in H and H' had been made, their ratio would have been found to vary considerably, indicating fluctuations in the value of γ . These fluctuations would be less marked than those reported by Emerson and Lewis, but qualitatively similar. According to them, the changes in γ which take place in the early part of a light exposure are compensated by corresponding changes in the beginning of the subsequent dark period. Warburg's addition of pressure changes derived from these two time intervals would be expected to minimize the evidence of fluctuations in γ .

Whether differences in the transition periods of the two curves shown in Figure 10.1 are due, as Warburg supposes, to purely physical differences in diffusion, or as we suggest, to fluctuations in γ , may be tested experimentally. According to Emerson and Lewis, the fluctuations in γ are due primarily to fluctuations in CO_2 exchange, while O_2 exchange follows the expected course in the sequence of light and dark periods. The manometric pressure changes will include O_2 exchange alone if the cells are suspended in a carbonate mixture which maintains CO_2 at constant partial pressure (85 parts $M/10$ NaHCO_3 , 15 parts $M/10$ K_2CO_3). In this medium we can compare the physical response of the 5 ml. and 8 ml. fillings without interference from possible irregularities in CO_2 exchange. Then, using the same cell suspension and suspending fluid, we can saturate with 8 per cent CO_2 in air. This converts all the carbonate to bicarbonate, and restores the pressure changes due to CO_2 exchange, and we can see their effect on the readings during the transition period.

To assure comparability of conditions of physical diffusion in our experiment and in Warburg's, we used vessels as nearly as possible the same in volume as his, filled with the same liquid volumes, 5 and 8 ml. Our shaking speed was 150 per minute, the same as Warburg's. He does not specify the amplitude beyond saying that it was "small." However, the light absorption is quite sensitive to amplitude of shaking. We found that with 100 cmm. cells (the quantity Warburg used), an amplitude greater than about 5 mm. gave such obvious difference in light absorption for the 5 ml. and 8 ml. fillings, that Warburg could hardly have regarded

the absorptions as equal. Therefore we used an amplitude of 5 mm., and believe that this must have given a close duplication of his shaking conditions. We also used incandescent light, filtered through a red glass transmitting only wave lengths longer than about 620 m μ (nearly the same as Warburg's red glass). We used 150 cmm.

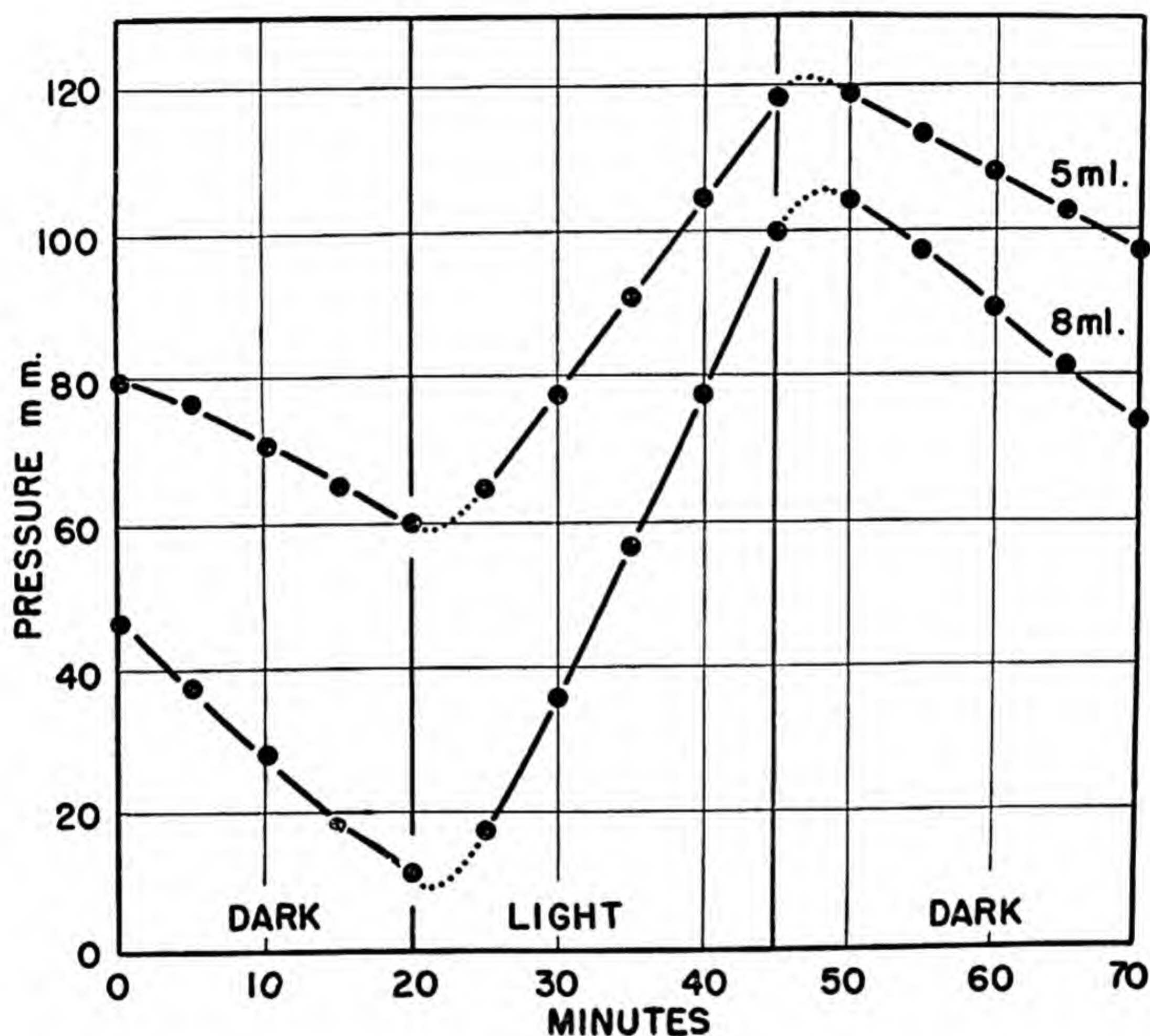


FIG. 10.2—Experiment to show shape of curves during dark—light transition period, when O_2 alone is responsible for pressure change. $20^\circ C.$, 150 cmm. cells suspended in carbonate mixture. Gas space = air. Incandescent light, filtered through red glass. The two sets of readings were made simultaneously. Vessel volume for 5 ml. fluid was 13.86 ml.; for 8 ml. fluid, 14.10 ml. Shaking 150 per minute, excursion 5 mm.

cells in each vessel, instead of the 100 cmm. which he used. This increase in quantity of cells gave somewhat larger pressure differences, and served to establish the course of the curves with greater certainty.

Figure 10.2 shows the results of a sequence of measurements in carbonate mixture. For both the 5 ml. and 8 ml. volume, the transition follows essentially the same course. There is no indica-

tion of immediate response for the 5 ml. filling, as indicated in Warburg's data plotted in Figure 10.1. In our opinion, the similarity of response shown in Figure 10.2 for the two different fluid volumes, makes it improbable that the differences shown in Figure 10.1 for the corresponding period are due, as Warburg supposes, to purely

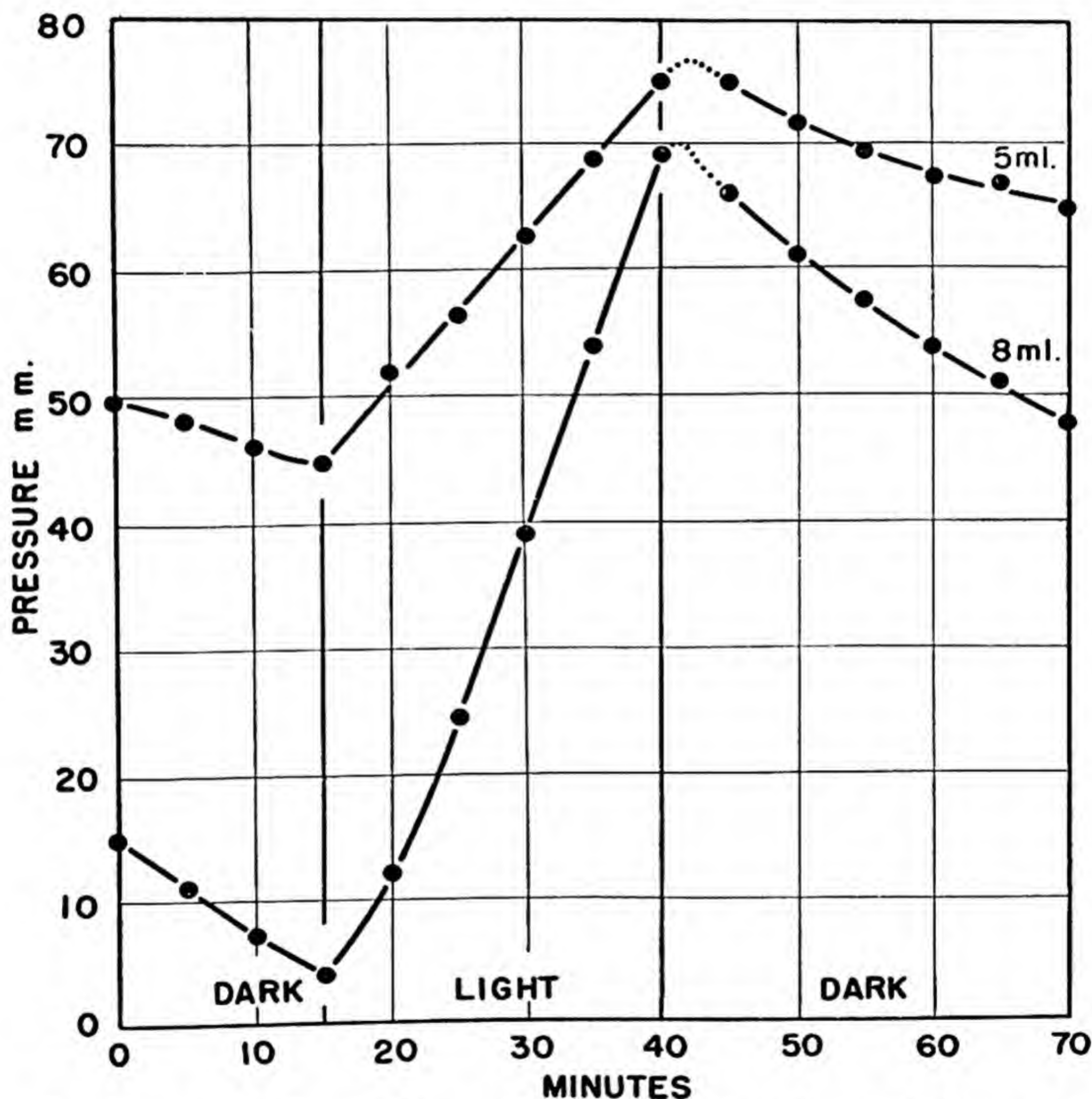


FIG. 10.3—Repetition of sequence of measurements shown in Figure 10.2, with same cell suspensions, but saturated with 8 per cent CO_2 in air, so that pressure changes represent both O_2 and CO_2 exchange.

physical effects; i.e., difference in rate of response to the change from dark to light. It remains possible that they indicate a temporary deficit in CO_2 consumption. If this is so, then by restoring CO_2 exchange to the carbonate mixture experiment, we should be able to duplicate the pattern of Warburg's observations.

Figure 10.3 shows a repetition of the same sequence of observa-

tions as in Figure 10.2, with the same cell suspension, but saturated with 8 per cent CO_2 in air, to restore pressure changes due to CO_2 exchange. The course of the curves during the transition period is now like the plot of Warburg's data. The transition from dark to light rate is gradual for the 8 ml. filling, but the 5 ml. filling shows immediate attainment of the new rate. Indeed, the rate for the first five minutes of light is slightly higher than the subsequent rate, though this difference might be regarded as within the limit of error. One could think of reasons why the inclusion of CO_2 exchange might further delay the response of the manometers, such as delay in attainment of steady states in carbonic acid—carbon dioxide equilibria, but there are no obvious reasons why it should accelerate response. Since differences in response to physical factors are too small to show up when O_2 alone is exchanged (Fig. 10.2), it is difficult to understand why a difference due to purely physical causes should appear in Figure 10.3, where CO_2 exchange is included, especially since the observed difference could only be interpreted as indicating increased speed of response.

We, therefore, attribute the straightening of the 5 ml. curve in Figure 10.3 not to physical effects, but to deficit in CO_2 consumption. This would have a greater effect in the case of the 5 ml. filling, since in the 8 ml. filling a larger fraction of the CO_2 exchanged remains dissolved in the fluid. We conclude from our observations that in the case of Warburg's experiments also, differences in physical diffusion must have been insufficient to justify the great difference in adjustment he made in the values of H and H' before computing γ . We note that his unadjusted values of H and H' lead to a ratio of 1.62, indicating a γ value of about -0.5 for the first ten minutes, instead of -0.93 , the value Warburg computed on the basis of the adjusted H and H' .

No great significance should be attached to the value of γ determined in this way, nor can Warburg's data be regarded as adequate proof of the existence of a CO_2 burst. The simple blood-gas manometers are inadequate for investigation of this problem. They are unsuited for the close study of rapidly changing rates of gas exchange because they cannot be shaken strongly enough to make the response of the manometer a satisfactory index of a changing rate of gas exchange in the cell suspension. In the ordinary course of research with simple blood-gas manometers, one would dismiss the minor differences shown in Figures 10.1, 10.2, and 10.3, as being beyond the reach of this experimental technique. One would compute gas exchange only from the pressure changes observed after steady rates had been attained. But since Warburg has used this method in an attempt to refute our claims, which were based on

a method far better adapted to the study of the phenomenon in question, we have felt under obligation to examine the results obtainable by Warburg's method, in the hope of discovering why he was unable to find any evidence of a phenomenon which, according to Emerson and Lewis, is of considerable magnitude.

Our first conclusion, from Figures 10.2 and 10.3, is that Warburg has inadvertently concealed the effect of the CO_2 burst by making adjustments in his data. But still it seems that the curves in Figure 10.1 suggest much smaller fluctuations in γ than those described by Emerson and Lewis. One would expect that if the CO_2 burst is large enough to account for an error of a factor of two in the quantum yield measurements, it should also be large enough to show up more clearly in the simple manometer measurements than the rather tenuous evidence we uncovered in Warburg's data.

There seem to be two reasons why his results might be expected to indicate only rather inconspicuous changes in γ . One is that he did not use cells comparable with those specially grown to give high quantum yield. The cells he used for γ determination show a very low rate of respiration, only about 0.20 cmm. O_2 per cmm. cells per hour at 20°C . His data for quantum yield measurements indicate a rate of respiration about 30 per cent greater, in spite of the lower temperature used for the efficiency measurements (10°C .). Properly, the measurements of γ should be made with cells of the same type as those used for the quantum yield measurements, and under the same environmental conditions.

A second reason for not expecting any clear evidence of CO_2 burst from Warburg's γ measurements, as well as from our own measurements plotted in Figure 10.3, is that very gentle shaking was used in order to avoid difference in light absorption for the large and small fluid volumes. Emerson and Lewis (6, p. 791) specify that in order to achieve sufficiently rapid gas exchange to make the CO_2 burst clearly evident, they used a shaking speed of 400 r.p.m., in a circular path 8 mm. in diameter. Besides this, they had a paddle dipping into the suspension to increase turbulence. The paddle is far more effective than the glass beads used by Warburg to improve mixing in his differential manometer vessel. Gas exchange was enormously accelerated compared to anything possible with the very gentle shaking described by Warburg for the γ measurements. Such rapid mixing is essential if significant readings are to be made at intervals of one minute or less, as was done by Emerson and Lewis. The CO_2 burst lasts only about a minute, and if five minutes elapse from the beginning of illumination to the first reading, the effect of the burst is largely concealed by the continuing photosynthesis. The ordinary blood-gas manometers with rectangular

vessels, such as Warburg used for his γ measurements, cannot be shaken hard enough to permit such rapid gas exchange. Very moderate shaking, with a fluid volume of 8 ml., causes fluid to splash into the entrance tube of the manometer.

However, if the large fluid volume is reduced from 8 to 7 ml.,

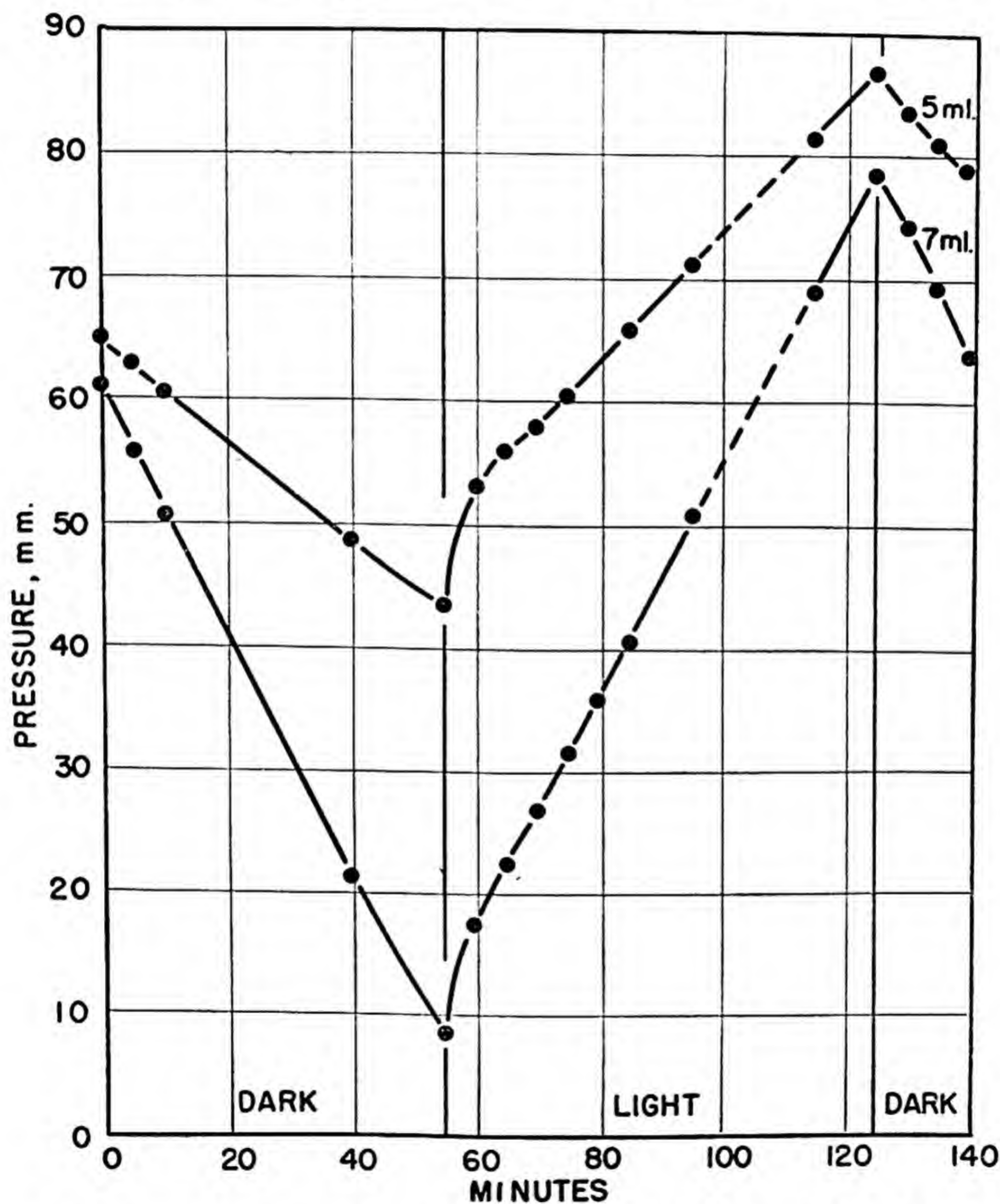


FIG. 10.4—Experiment to show effect of increased shaking on shape of curves during transition period. 20°C., 150 cmm. cells suspended in culture medium saturated with 8 per cent CO_2 in air. Incandescent light, filtered through red glass. The two sets of readings made simultaneously. Vessel volume for 5 ml. fluid was 14.10 ml., for 7 ml. fluid, 12.71 ml. Shaking 150 per minute, excursion 10 mm. Broken lines indicate ten-minute interval when vessels were allowed to stand unshaken to test for totality of light absorption.

the amplitude can be increased to 10 mm. without splashing fluid into the manometer tube. The response of the system to changing gas exchange is sufficiently accelerated so that unambiguous evidence of the CO_2 burst is obtainable, even with the simple manometers. The 10 mm. shaking amplitude, at a speed of 150 per minute, gives a considerable difference in light transmission between the 5 ml. and 7 ml. filling, even when 150 cmm. cells are used in each vessel. This makes precise computation of γ impossible, but the question at issue is whether or not γ is constant. Since any difference in light absorption in the two vessels will remain constant during the experiment, the ratio H'/H will be an index of the constancy of γ , even though the precise value of γ may not be established.

Figure 10.4 shows a sequence of measurements made with increased shaking. The cells were grown in the manner prescribed by Warburg for obtaining high quantum yields (reduction in light intensity after initial growth at high intensity). Emerson and Lewis (5) noted that this procedure gave cells with prominent CO_2 burst. For the experiment shown in Figure 10.4, such cells were centrifuged from their culture medium, washed twice in fresh medium, saturated with 8 per cent CO_2 in air, and suspended in the same medium for filling into the manometer vessels. After the vessels were connected to their manometers, the gas spaces were swept out with 8 per cent CO_2 in air. Under these conditions, pressure changes represent the sum of both O_2 and CO_2 exchange.

The readings plotted in Figure 10.4 show a sudden and temporary increase in rate of pressure change immediately following the change from dark to light, for both the 5 ml. and 7 ml. fillings. By reducing the larger fluid volume from 8 to 7 ml., and increasing the amplitude of shaking from 5 to 10 mm., we have improved the response during the transition period enough so that there is no longer any initial deficit of positive pressure such as Warburg observed for his 8 ml. filling.

Both fillings show an excess of pressure, rather than a deficit, during the transition period. This excludes the interpretation which seemed plausible to Warburg, and leaves no basis for a differential adjustment of the observed pressure changes. We may, therefore, compute γ and O_2 - CO_2 exchange from the unadjusted values. We must, however, bear in mind the reservation mentioned above that (apart from small differences in diffusion) H and H' for the light period may not be strictly comparable. This is because, with the stronger shaking, more light is transmitted by the 5 ml. filling, and therefore less is absorbed, than by the 7 ml. filling. However, we have applied Warburg's test of equality of light absorption—letting the vessels stand unshaken for five minutes (dotted portion of curves), and then restoring shaking. This does not alter the slopes

of the two curves appreciably, so the loss of light absorption from shaking cannot be large.

The values of H and H' for the first ten minutes of light are $+12.6$ and $+14.4$ mm., respectively. For the ten-minute dark just before the light, the corresponding values for respiration are -3.5 and -8.8 mm. We subtract the dark pressures from the light pressures, and find the pressures attributable to the light exposure:

$$\begin{aligned} H &= +16.1 \\ H' &= +23.2 \end{aligned}$$

For H , $K_{O_2} = 0.864$, and $K_{CO_2} = 1.292$. For H' , $K'_{O_2} = 0.554$, and $K'_{CO_2} = 1.153$. From these constants we compute the O_2 and CO_2 exchange, and γ , using the same equations Warburg used:

$$\begin{aligned} X_{O_2} &= +10.2 \\ X_{CO_2} &= +5.5 \\ \gamma &= \frac{X_{CO_2}}{X_{O_2}} = +0.54. \end{aligned}$$

Taking the values from the ten-minute light interval just before the dotted parts of the curves, H and H' are $+5.2$ and $+10.2$. Using the same respiration values as above (-3.5 and -8.8), we find the pressures attributable to the light:

$$\begin{aligned} H &= +8.7 \\ H' &= +19.0, \end{aligned}$$

and multiplying by the appropriate constants,

$$\begin{aligned} X_{O_2} &= +18.3 \text{ cmm.} \\ X_{CO_2} &= -16.1 \text{ cmm.} \\ \gamma &= -0.88 \end{aligned}$$

Our dark pressure changes are larger than Warburg's, because we used more cells, and our cells also had higher respiration. It is, therefore, worth while to compare the respiratory quotients just before and just after the light exposure. The values of H and H' for the ten minutes preceeding the light exposure are -3.5 and -8.8 mm. From these we find:

$$\begin{aligned} X_{O_2} &= -9.64 \text{ cmm.} \\ X_{CO_2} &= +9.90 \text{ cmm.} \\ \gamma &= -1.03. \end{aligned}$$

For the first ten minutes following the light exposure, H and H' are -5.8 and -9.6 , giving:

$$\begin{aligned}X_{O_2} &= -6.11 \text{ cmm.} \\X_{CO_2} &= +2.20 \text{ cmm.} \\\gamma &= -0.36.\end{aligned}$$

All these figures are in good agreement with the results of Emerson and Lewis. At the end of the long dark period, just before the beginning of the light exposure, γ has attained a value of -1.03 . Illumination causes both O_2 and CO_2 evolution, and a temporary change in γ to $+0.54$. After thirty minutes of illumination, γ has become -0.88 . Darkening changes it to -0.36 , indicating a deficit in CO_2 production. Emerson and Lewis noted that the change in value of γ at the beginning of the dark period was less extreme than at the beginning of the light period, but that in darkness the return to a value close to -1 was slower than in light. All this is as fully confirmed by the data for Figure 10.4 as could be expected, considering the shortcomings of the simple manometers for measurement of rapidly changing rates.

Warburg's manometric γ determinations lead to qualitatively similar results if no adjustments are made in the readings. Our Figures 10.2 and 10.3 show no basis for his adjustment of manometer readings before computation of γ . Quantitatively, Warburg's deviations are small, and we attribute this to the fact that for his γ determinations he used cells different from those grown especially for his quantum yield measurements, and therefore probably incapable of showing such a large CO_2 burst.

Emerson and Lewis emphasized that, in spite of the violent stirring of the cell suspension, there must still have been a finite lag between changes in rate of gas exchange within the cells and registering of the change on the manometer. More rapid methods of following this exchange have been tried, for instance by Aufdemgarten (2), Blinks and Skow (3), and McAlister and Meyers (9). The results of these investigators abundantly confirm our finding that illumination results in an initial deficit in CO_2 consumption or actual positive production of CO_2 . But each of these methods has other disadvantages for the further investigation of the CO_2 burst. The methods of McAlister and of Aufdemgarten are only suitable for low partial pressures of CO_2 . Emerson and Lewis found that relatively high partial pressures (from 1 to 5 per cent in air) are necessary to make the burst large enough to be clearly evident. The method of Blinks and Skow is very sensitive, but does not lend itself to measurement of exchange in absolute units. So far, the differential manometer has given us the best approach to the study of this phenomenon.

Warburg attributes our observations with the differential mano-

meter to spurious effects from the formation and bursting of foam bubbles during the violent shaking. This interpretation is not in accordance with the data. If a bubble forms or bursts, and thereby causes an error in one reading, the error is compensated at the next reading. As shown in Figure 10.4, we are dealing with a sustained pressure increase, which does not collapse like a bursting bubble. We are familiar with the effects of foaming, which, as Warburg says, may be a disturbing factor. But these effects are minuscule compared to the pressures caused by the CO_2 burst. Also, the bursts are perfectly correlated with the start of illumination, while the effects of bubble formation are random. Besides this, the building up of foam has the opposite effect to the CO_2 burst. We have put soap solution in the manometer vessel, and followed the pressure changes while foam was built up as a result of shaking. Foam building resulted in gradual diminution of pressure, due to compression of the gas in the vessel by the surface tension of the foam bubbles. We observed no sudden increases in pressure such as were associated with the CO_2 burst, and we can think of no way in which foaming could cause such increases.

BEARING OF THE γ MEASUREMENTS ON THE QUANTUM YIELD PROBLEM

Warburg emphasized the improbability that changes in γ should go undetected, and still be large enough to account for an error of a factor of 3 in the quantum yield. In discussing Figures 10.2, 10.3, and 10.4 we have explained how the changes in γ escaped detection in Warburg's measurements. Some of the same data can be used to show the extent of error to be expected in the quantum yield from a given change in γ . If we were to assume, as Warburg did, a constant value of -0.93 for γ , then we could compute rate of photosynthesis from the data of Figure 10.4, using either the 5 ml. or the 7 ml. filling. The vessel constant for O_2 exchange for a single vessel in which both O_2 and CO_2 are being exchanged is

$$K_1 = \frac{k_{\text{O}_2} \cdot k_{\text{CO}_2}}{k_{\text{CO}_2} + \gamma \cdot k_{\text{O}_2}}.$$

For the 5 ml. filling, $K_1 = 2.285$, and for the 7 ml. filling $K'_1 = 1.002$.

In the preceding section we have shown that in the first ten minutes of light and the first ten minutes of darkness, γ deviates greatly from -1 ($+0.54$ and -0.36 respectively). Just before the beginning of light and in the middle of the light period, γ is close to -1 (-1.03 and -0.88 respectively). The time intervals where the value of γ is far from -1 correspond roughly to the intervals Warburg used for measuring quantum yields, and the periods where

γ is close to -1 correspond to the intervals which Emerson and Lewis chose in order to avoid errors from fluctuations in γ . For the 7 ml. filling, the pressure changes for the first ten minutes light and first ten minutes dark immediately following the light period are $+14.4$ and -9.6 . Subtracting the dark from the light pressure gives a value of $H' = +24.0$ mm., the pressure change due to photosynthesis. Photosynthesis computed on Warburg's assumption that $\gamma = 0.93$ is

$$H' \cdot K_1' = +24.0 \times 1.002 = +24.0 \text{ cmm. O}_2.$$

If we compute from the ten-minute dark just before the light exposure, and from the ten-minute light just before the dotted part of the curve (γ close to -1 in each case), we find $H' = +10.2 - (-8.8) = +19.0$ and computed photosynthesis is

$$H' \cdot K_1' = +19.0 \times 1.002 = +19.0 \text{ cmm. O}_2.$$

Making the same calculations from the same time intervals for the 5 ml. filling, we find the computed photosynthesis to be:
Ten-minute intervals when γ is far from -1 :

$$H \cdot K_1 = +18.4 \times 2.285 = +42.0 \text{ cmm. O}_2.$$

Ten-minute intervals when γ is close to -1 :

$$H \cdot K_1 = +8.7 \times 2.285 = +19.95 \text{ cmm. O}_2.$$

For the intervals when γ is close to -1 , both the 5 ml. and 7 ml. fillings give the same (correct) value for photosynthesis, 19.0 or 19.95 cmm. O₂. For the time intervals when γ is not close to -1 , the deviation from correct value is much greater in the case of the 5 ml. filling, being larger by a factor greater than 2. For a given error in the assumed value of γ , the error in the computed photosynthesis will be greater, the larger the value of K_1 . According to Warburg, the vessel volume and fluid volume used for his quantum yield measurements led to a value of 5.40 for K_1 , almost twice as large as K_1 for our 5 ml. filling. This could easily lead to an error of a factor of 3 or 4 in the computed photosynthesis. Actually, a factor of 2 or $2\frac{1}{2}$ would be sufficient to account for the difference between Warburg, and Emerson and Lewis. Warburg mentions a quantum requirement of 12 found by Emerson and Lewis, compared with his own value of 4, but many of Emerson and Lewis's measurements led to values close to 10, with occasional values approaching 8. Rieke's results are similar.

Our comparison of the error resulting from the use of an incorrect γ value in computing photosynthesis for the 5 ml. and 7 ml. fillings emphasizes the importance of measuring γ under the same

conditions as are used for measuring the quantum yield. Warburg not only used very different gas-to-liquid volume ratios, but also different temperatures and gas mixtures. For the γ measurements he specifies 20°, and 10 per cent CO_2 in O_2 ; for the quantum yields, 10° and 5 per cent CO_2 in air. As we mentioned in discussing the γ measurements, the cells he used for γ determination showed a much lower rate of respiration than the cells for his yield determinations. Emerson and Lewis, on the other hand, made the two determinations under identical conditions, with the same cell material, so their γ measurements were strictly comparable with their quantum yield determinations.

DISCUSSION OF WARBURG'S NEW QUANTUM YIELD MEASUREMENTS

Little need be said of these new determinations, since his method differs in no essential respect from that used by Warburg and Negelein twenty-five years ago. He finds again a quantum requirement of about 4 per molecule of O_2 produced. He shows a table of data, and four figures illustrating the sequence of measurements. In each case he gives figures for the quantum yield calculated from the total time of illumination, and from the so-called stationary conditions. He says that within the limit of error, the yields are the same by each method of calculation.

However, we note that with one exception (13, Fig. 4) the value for total time of illumination is always lower than for "stationary conditions." This regularity was also noted by Rieke, and by Emerson and Lewis, and it was this which first caused Emerson and Lewis to question the reliability of their own measurements made by Warburg and Negelein's method. Warburg's new comparison of the results by the two methods of computation would be inconclusive, were it not for the fact that the discrepancy is always in the same direction, and also that in one case (Warburg's Fig. 6) the difference is so large that Warburg himself refers to it as the "Emerson effect." He found this effect in the one experiment reported at a light intensity high enough to give photosynthesis substantially above compensation of respiration. This is in agreement with Emerson and Lewis, who noted that below compensation the CO_2 burst was so inconspicuous that improvements in the manometric technique were required to make it evident.

In Warburg's Figures 3, 4, and 5, where quantum yields were measured near or below compensation, there is no obvious evidence of temporary deviation from steady rates. However, the fifteen-minute intervals were too short to show whether steady rate had been attained, and the shaking was too gentle to bring out the effect of the transient changes in γ . The pressures due to these changes

were spread out over the entire fifteen-minute periods. Either faster shaking or longer periods would be required to confirm the findings of Emerson and Lewis. But inspection of Warburg's table of data for quantum yield measurement shows that both the initial dark readings and the initial light readings tend to deviate slightly from steady rate, in the direction that would be accounted for by the fluctuations in γ revealed in our experiments. This, together with the fact that Warburg regularly finds a slightly higher yield computed from total time than from so-called stationary conditions, leads us to believe that the pressure changes produced by his algae were the result of fluctuations in γ essentially the same as those we have described, and that his measurements, if correctly interpreted, would lead to quantum yields in agreement with ours.

Warburg attributes the "Emerson effect" shown in his Figure 6 to foaming, but, as we explained in connection with the γ measurements, the forming and bursting of bubbles does not lead to sustained increases in pressure.

SUMMARY

Warburg has reported manometric measurements of the ratio of $\text{CO}_2\text{-O}_2$ exchange, which refute the findings of Emerson and Lewis that the exchange ratio is subject to wide variations. According to Emerson and Lewis, these variations had caused errors in Warburg and Negelein's earlier computation of quantum yield of photosynthesis. Warburg reports that the exchange ratio is constant, and reaffirms his claim that the quantum requirement per molecule of oxygen evolved in photosynthesis is 4.

We have checked Warburg's new measurements, and found them inadequate for the demonstration of variability of gas exchange ratio under the conditions of the quantum yield measurements. Improvement of Warburg's technique leads to unmistakable evidence of fluctuations in exchange ratio like those described by Emerson and Lewis. The observed magnitude of the fluctuations is sufficient to account for the difference between the quantum yields reported by Warburg, and by Emerson and Lewis.

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11.

Quantum Efficiency of Photosynthesis in *Chlorella*

The Respiration Correction and Dependence of Quantum Yield on the Wave Length of Light

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IN DETERMINING the quantum efficiency of photosynthesis the amount of oxygen evolved or of carbon dioxide absorbed by the green plant exposed to light must be corrected for the continuing process of respiration. The amount of oxygen and carbon dioxide exchanged in respiration is determined during each dark period immediately preceding and following the light period, or it is determined during only one of these dark periods. If the rate of respiration during photosynthesis in the light is not actually the same as that determined in the dark, or if there is an anomalous exchange of one or both of the two gases involved in the measurements during either the light or dark period (1), the respiration correction becomes an important source of error.

In the experiments reported here, the amount of photosynthesis has been obtained by determining the oxygen produced using the dropping mercury electrode (2). The experiments were designed so that quantum efficiencies could be determined when the correction for respiration was applied in the usual manner and also when the need for this correction, as such, was eliminated. The quantum efficiency obtained in either case was essentially the same, namely, 0.10–0.08, for red, green, blue, and violet light.

The quantum efficiency obtained agrees with the most generally accepted value for the photosynthetic process. However, the value

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of 0.25 originally reported by Warburg and Negelein (3), while never having been confirmed in these laboratories (4, 5, 6, 7), nor in some others (8, 9), has nevertheless received support from Eichhoff (10), and recently Warburg (11, cf. also 12) has again reported a value of 0.25 obtained with the manometric method. The results presented in this communication are offered, in addition to the experimental evidence previously published, in support of the lower value for the quantum efficiency of photosynthesis, that is, 0.10–0.08, within the wave lengths designated.

EXPERIMENTAL

The plant material consisted of suspensions of the unicellular alga *Chlorella pyrenoidosa*. This alga was maintained in pure culture and propagated as stock on a solid medium (Medium No. 1). In providing sufficient quantities of cells for the quantum yield determinations the alga was propagated in a liquid medium (Medium No. 2). The composition of the media was as follows:

Medium No. 1		Medium No. 2	
KH ₂ PO ₄	0.25 gm.	KH ₂ PO ₄	0.018 M
MgSO ₄ · 7H ₂ O	0.25 gm.	K ₂ HPO ₄	0.0058 M
CaCl ₂	0.25 gm.	MgSO ₄	0.02 M
NaNO ₃	0.25 gm.	KNO ₃	0.025 M
Sucrose	2.00 gm.	FeSO ₄	0.00001 M
Peptone	4.00 gm.	A-Z solution	1.0 ml.
Agar	15.0 gm.	Distilled H ₂ O to	1.0 L.
Distilled H ₂ O to	1.0 L.		

The A-Z solution used routinely consisted of the following salts dissolved in 18 liters of distilled water containing 30 ml. of 18 N HNO₃: AlKSO₄, 2.7 gm.; KI, 0.5 gm.; KBr, 0.5 gm.; Na₂TiO₃, 1.0 gm.; SnCl₂ · 2H₂O, 0.5 gm.; LiCO₃, 0.4 gm.; MnCl₂ · 4H₂O, 7.0 gm.; H₃BO₃, 11.0 gm.; ZnSO₄, 1.0 gm.; CuSO₄ · 2H₂O, 1.0 gm.; NiSO₄(NH₄)₂SO₄ · 6H₂O, 1.5 gm.; Ca(NO₃)₂ · 4H₂O, 1.0 gm.; (NH₄)₆MO₇O₂₄ · 4H₂O, 0.5 gm.; Na₃VO₄, 0.5 gm.; K₂Cr₂O₇, 0.2 gm. The pH of the liquid medium was 5.76 at 25°C.

The liquid cultures, inoculated with algal cells from stock test-tube cultures, consisted of 150 ml. portions of liquid medium contained in 250 ml. Erlenmeyer flasks. These were aerated by bubbling through the medium of each flask air containing 5 per cent CO₂. The lower 3–4 cm. of the flasks were immersed in a glass-bottomed water bath at 22°–23°C., and illuminated from below with Mazda lamps (4) — (39,500 ergs/cm.²/sec. total radiation). The alga

multiplied rapidly under these conditions, and in six to nine days the quantity of cells in a single flask was sufficient for an experiment.

Each determination of the quantum yield of photosynthesis was carried out in a room which was maintained at a constant temperature during the course of the experiment. The range of temperatures for the different experiments was 24.6° – 28.6° C. Oxygen produced in photosynthesis and consumed in respiration was determined with the dropping mercury electrode essentially in the manner described by Petering, Duggar, and Daniels (5). The algal cell suspension, obtained directly as a liquid culture, was removed from the culture flask and immediately pipetted into the irradiation-reaction vessel.

The work at this laboratory has already shown that the activity of this alga is not affected by the anode pool of mercury at the bottom of the irradiation-reaction vessel. However, in order to insure constancy of distance between the cathode tip and the anode the vessel was modified to the form shown in Figure 11.1. With this type of vessel there is considerably more uniformity in the current flow in the galvanometer circuit than with the cell described by Petering, Duggar, and Daniels (5). The vessel, 4 cm. in cross section and 1.4 cm. in thickness, was constructed of pyrex glass with optically flat Corex windows 1.0 cm. apart. The glass capillary tip of the cathode was drawn out and cut off so that when ground into the neck of the vessel it projected down to a point about 5 mm. from the center of the vessel and midway between the front and back windows.

The irradiation vessel-dropping Hg-electrode assembly, or reaction vessel, was calibrated for dissolved oxygen as described by Petering and Daniels (2). Liquid culture medium from algal

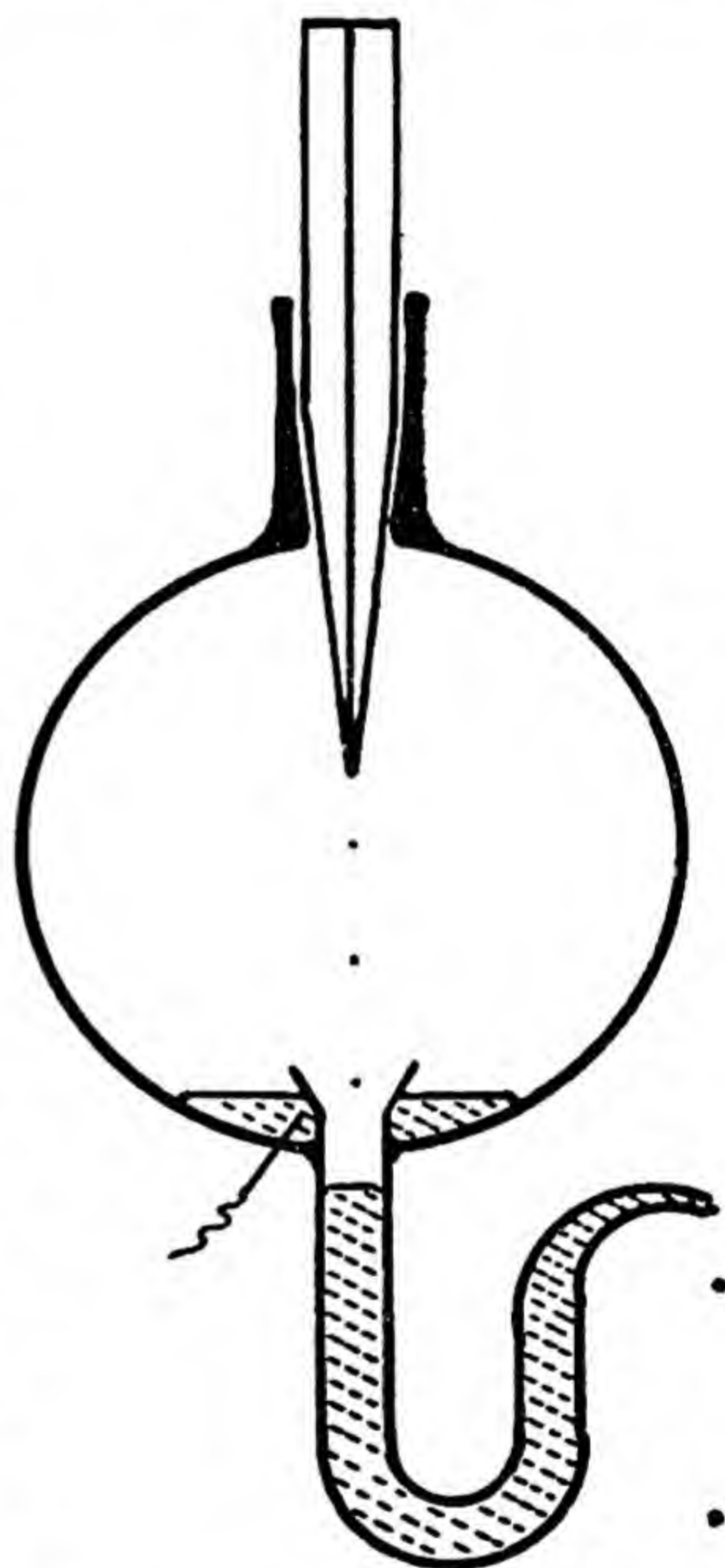


FIG. 11.1—Reaction vessel with stable anode-pool of mercury.

cultures of the same age as those used as the source of the algal cell suspensions was used in this calibration; the calibration was also extended to cover the temperature range of the experiments.

The light sources were a tungsten filament projection lamp (500 watt) and a G. E. AH-6 water-cooled mercury arc. To obtain various intensities from these sources the current to the former was regulated by a series resistance, while for the latter the intensity was reduced when desired by interposing screens of fine-mesh brass wire in the beam. In the experiments to be described the following wave lengths were used: 4,047-78, 4,358, and 5,461 Å, isolated by means of filters from the spectrum of the mercury arc, and a band in the red portion of the visible spectrum having a maximum of 6,300 Å and an average wave length for quantum calculations of 6,500 Å, isolated by means of filters from the spectrum of the tungsten filament source. The filter combinations were the same as those described by Dutton and Manning (7) with the exception that the filters used in isolating the 6,500 Å band consisted of 5 cm. N/20 CuSO_4 , 20 cm. H_2O , and Corning No. 243 (2.37 mm.) glass filter.

Energy distribution curves for the filter combinations, determined spectrophotometrically, showed that the violet, blue, and green radiations were essentially monochromatic (98 per cent or better). In the red band, isolated from the spectrum of the tungsten filament lamp, 84.5 per cent of the radiation was in the region 6,000-7,500 Å and the remaining 15.5 per cent of longer wave lengths. While it is doubtful that the algal cells use any considerable fraction of the radiation above 7,500 Å in photosynthesis (8), the transmission of radiation by the algal cell suspension was found to rise rapidly as the wave length of the radiation increased from 8,000 Å. The error in the determination of quantum yields for red light would therefore be less than that supposedly introduced by assuming all of the radiation isolated from the tungsten source to be used in photosynthesis. Furthermore, in the experiments to be described a large fraction of the incident radiation was transmitted by the algal cell suspension. The transmitted radiation contained a greater percentage of its energy as wave lengths longer than those active in photosynthesis than did the incident radiation. Thus it appears that the error introduced in calculating the quantum yields for red light could not be greater than 10 per cent.

The arrangement of light sources, filters, lenses, reaction vessel, and thermopile is shown in Figure 11.2. The algal cell suspension could be placed in darkness, illuminated with light of a single color, or illuminated with red light along with either violet, blue, or green light. Intensity measurements were made with a large area (2 cm.²)

thermopile. This instrument, which was calibrated with a radiation standard supplied by the U. S. Bureau of Standards and with uranyl oxalate (for 4,358 Å), had an absolute sensitivity of 81.6 ergs/cm.²/sec./cm. scale deflection of the galvanometer. Loss of light through reflection from the windows of the reaction vessel and thermopile were determined, and suitable correction factors applied in calculating the quanta of light absorbed by the algal cells.

It was found that the amount of light absorbed by the nutrient solution was approximately 1 per cent for the red and less than 1 per cent for the other wave lengths, as had been previously deter-

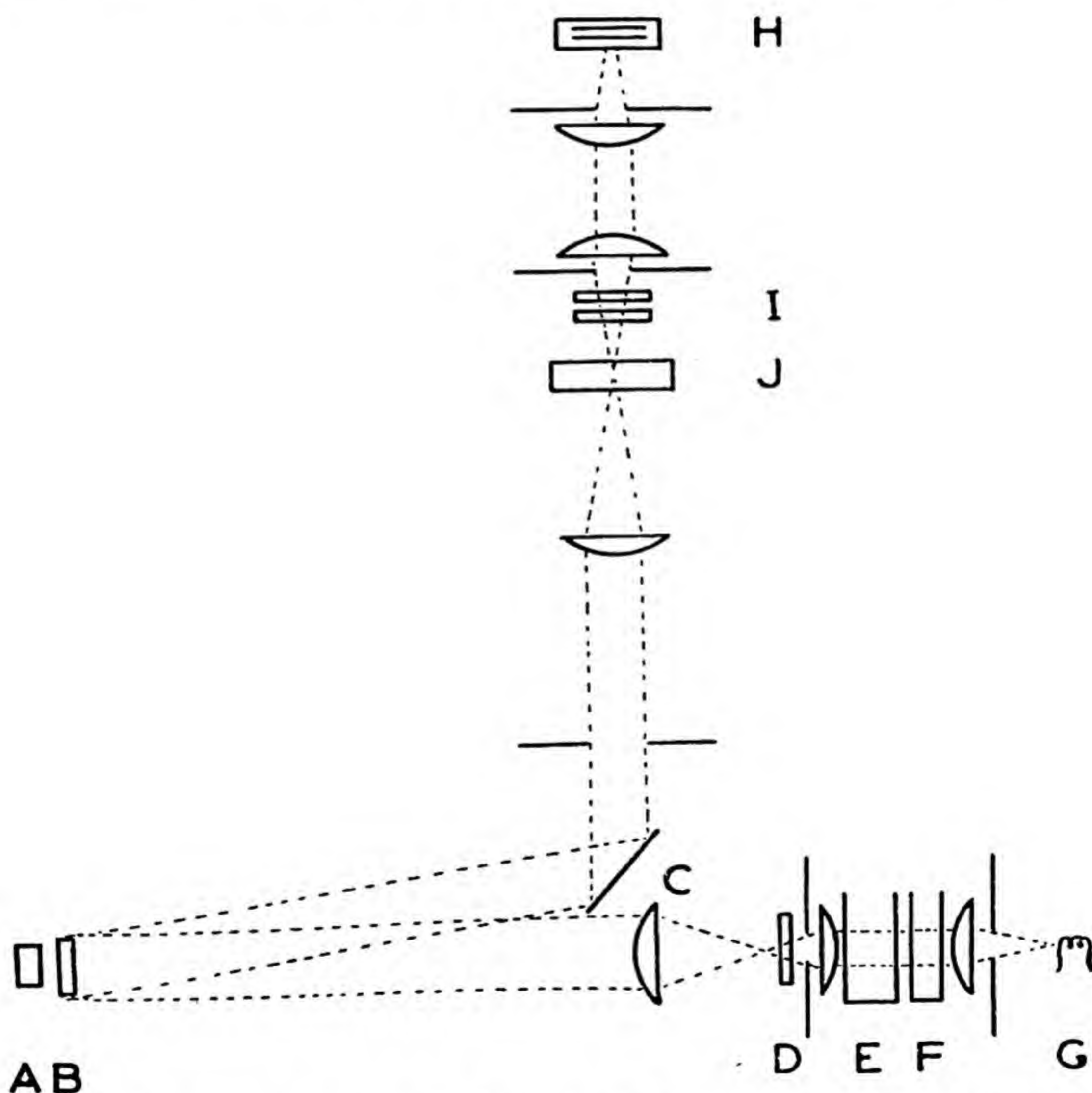


Fig. 11.2—Optical system. A, thermopile; B, reaction vessel; C, mirror; D, glass filter; E, water; F, N/20 CuSO₄ solution; G, tungsten filament lamp; H, mercury arc; I, glass filters; J, N/20 CuSO₄ solution.

mined by Dutton and Manning (7). The beams of light from both sources were parallel and larger in cross-sectional area than the reaction vessel; moreover, both were approximately of uniform intensity over the cross-sectional area which was intercepted by the reaction vessel. Light from either source struck the front window of the reaction vessel at an angle of 8° from normal incidence in a plane common to both light beams. Light transmitted by the algal cell suspension was measured with the thermopile placed directly behind the rear window of the reaction vessel, where it would intercept light passing through the suspension immediately below the tip of the cathode.

Since the receiver of the thermopile was about 1 cm. behind the window of the thermopile, it was thought that some rays of light scattered by the algae might pass through the thermopile window at angles such that they would not impinge on the receiver. However, little difference in the intensity of the transmitted light was found regardless of whether the measurements were made with this thermopile, or with one of almost identical construction equipped with a highly polished platinum-foil reflector, lining the passage between the window and the receiver to reflect the scattered rays onto the receiver. Little difference was observed whether either of these two thermopiles was clamped to the rear window of the reaction vessel or placed at distances of 1 and 3 cm. behind it. On the basis of these results it was concluded that no significant error in determining the energy absorbed was introduced by the scattering of light by the algal cells. The constancy of the intensity of the incident light was monitored by frequently measuring the transmitted light. The intensity of the incident light was measured before the initial dark period and at the end of the final irradiation period.

In determining the quantum yields, an algal cell suspension from a culture flask already saturated with air containing 5 per cent CO_2 was pipetted into the reaction vessel. The rate of respiration was then determined over a period of time while the algal cells were in the dark. A shutter in the light train was then opened and the apparent rate of photosynthesis (respiration and photosynthesis) determined while the algal cells were exposed to light of a particular color. Following the light period, respiration in darkness was again determined, or the light intensity was increased by additional light of another color and the apparent rate of photosynthesis again determined. The average rate of decrease in the concentration of dissolved oxygen during respiration for each period immediately preceding and immediately following the respiration-photosynthesis period was added to the rate of increase in concentration of dissolved oxygen during the time the algal cells were irradiated. Thus the rate of oxygen production due to photosynthesis alone

was obtained. In those instances where the oxygen concentration decreased during respiration-photosynthesis, this rate of decrease in oxygen concentration was subtracted from the rate of decrease in oxygen concentration during respiration. For those periods when the algal cells already exposed to light of a particular color were subjected to more light of a different color, the rate of photosynthesis due to the additional light was determined as the rate of increase in dissolved oxygen during exposure to the two colors of light above the rate during exposure to the single color of light.

The duration and sequence of the light and dark periods along with changes in the dissolved oxygen concentration for four experiments are presented in Figure 11.3. The method of calculating quantum yields is detailed in Table 11.1. Quantum yields for one color alone and for additional light of a different color in two-color combinations are presented in Table 11.2.

DISCUSSION

The data and quantum yields presented in Table 11.2 are from experiments chosen because they were satisfactory from the following standpoints: (1) there was no detectable change in the

TABLE 11.1
QUANTUM EFFICIENCY OF PHOTOSYNTHESIS AT 6,500 Å

Oxygen released by algal cells (20 minute light period):	
(1) Sensitivity of dropping Hg-electrode, as molecules of O_2 per $cm.^3$ per cm. galvanometer scale deflection (28°C.):.....	0.74×10^{16}
Changes in O_2 concentration as rate of change in the deflection of the galvanometer:	
(2) Respiration	-0.115 cm./min.
(3) Respiration and photosynthesis	0.065 cm./min.
(4) Photosynthesis (2) + (3)	0.18 cm./min.
(5) Photosynthesis for 20 minutes (4) \times 20	3.6 cm.
(6) Molecules of O_2 released in photosynthesis by 1.0 $cm.^3$ of the algal cell suspension (5) \times (1)	2.66×10^{16}
Quanta absorbed by algal cells (20 minute light period):	
(7) Absolute sensitivity of thermopile per cm. deflection of the galvanometer	81.6 ergs/ $cm.^2$ /sec.
Energy absorbed by 1.0 $cm.^3$ of the algal cell suspension, corrected for losses at thermopile window and in irradiation vessel:	
(8) Incident intensity	1,188. ergs/ $cm.^2$ /sec.
(9) Transmitted intensity	571. ergs/ $cm.^2$ /sec.
(10) Energy absorbed by algal cells (8) - (9)	617. ergs/ $cm.^2$ /sec.
(11) Energy absorbed in 20 minutes (7) \times (10) \times 1,200 ..	7.40×10^6 ergs
(12) Quanta per erg of 6,500 Å radiation	3.31×10^{11}
(13) Quanta absorbed by algal cells in 20 minutes (11) \times (12)	2.45×10^{17}
Quantum efficiency (6)/(13)	0.108

concentration of algal cells (15–20 million cells per cm.³) in the vicinity of the cathode of the dropping mercury electrode; (2) no difficulties were encountered in maintaining the light intensity, or intensities, constant during each of the irradiation periods; and (3) respiration remained relatively unchanged during successive dark periods.

By propagating the alga as described and by using relatively young liquid cultures directly as suspensions in the reaction vessel,

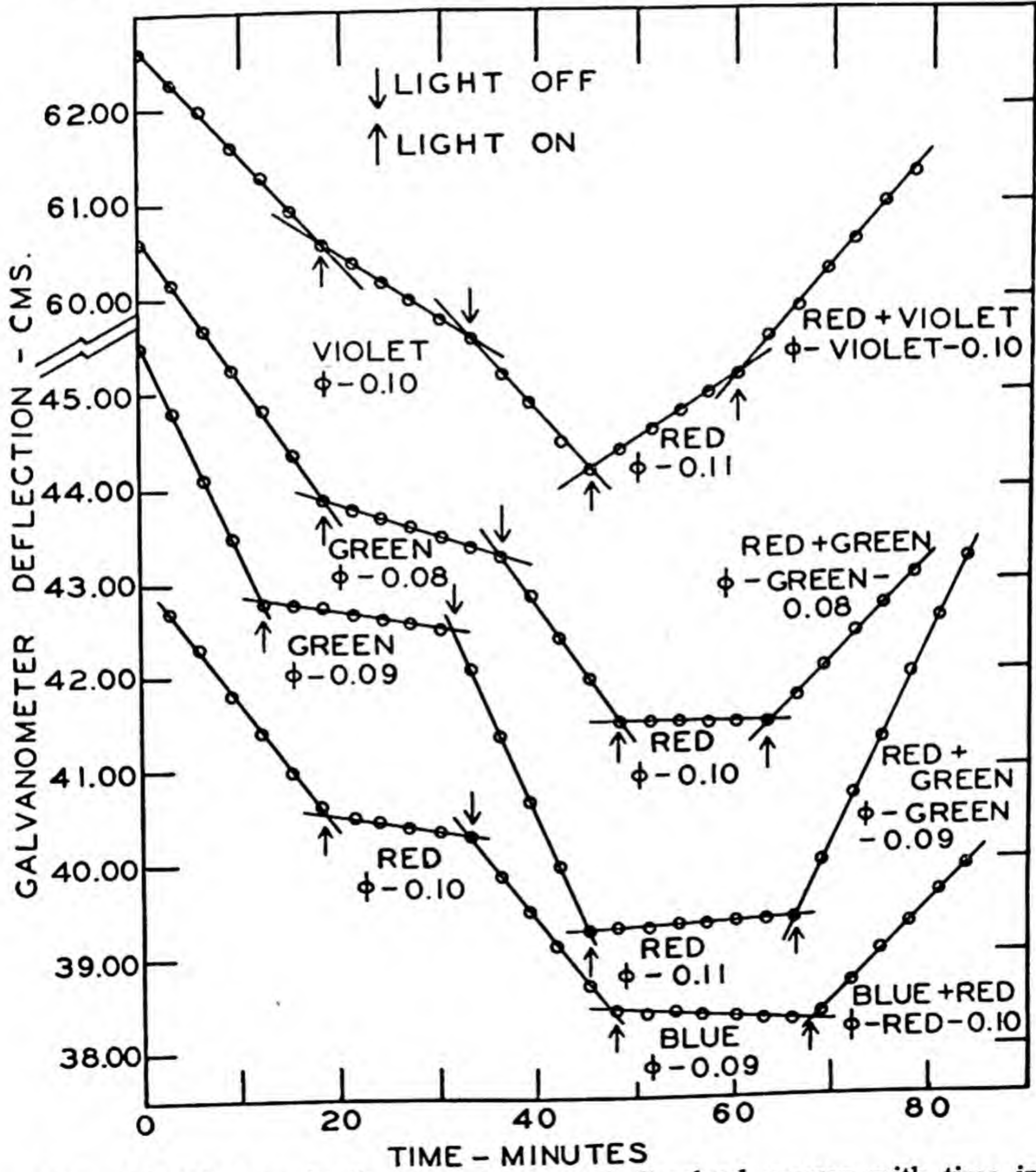


FIG. 11.3—Changes in the concentration of dissolved oxygen with time in algal cell suspensions in light and in darkness, and the quantum efficiencies obtained.

there was no detectable change in algal cell concentration (as a change in the percentage of light transmitted) in the vicinity of the capillary tip of the dropping mercury electrode. In those instances where considerably older cultures (twelve to eighteen days) were tried as suspensions, the cells apparently differed among themselves as to specific gravity as well as to size, since they settled out at different rates. However, all cells settled out at nearly the same rate in suspensions of cells from young cultures (six to nine days). The production or utilization of oxygen by the algal cells is determined as changes in concentration of dissolved oxygen in the vicinity of the capillary tip of the dropping mercury electrode. Thus, when the algal cells all settle out at approximately

TABLE 11.2
QUANTUM YIELDS OF PHOTOSYNTHESIS FOR SINGLE AND SUPERIMPOSED
WAVE LENGTHS OF LIGHT

Exp. No.	Composition of Incident Light	Intensity ergs/cm. ² /sec.	Absorption Percentage	Quantum Yield
1.....	6500 Å	1958	76	0.074
	4358	1575	100	0.11
	{6500	{1958	{76	0.11(Blue) *
	{4358	{1575	{100	
2.....	6500	783	46	0.12
	4358	983	74	0.11
	{4358	{983	{74(Red) †
	{6500	{783	{46	
3.....	6500	1180	50	0.11
	4358	1288	91	0.09
	{4358	{1288	{91	0.10(Red)
	{6500	{1180	{50	
4.....	5461	2532	40	0.08
	6500	1610	49	0.10
	{6500	{1610	{49	0.08(Green)
	{5461	{2532	{40	
5.....	5461	2437	64	0.09
	6500	1392	74	0.10
	{6500	{1392	{74	0.09(Green)
	{5461	{2437	{64	
6.....	4047-78	460	77	0.10
	6500	1188	52	0.11
	{6500	{1188	{52	0.10(Violet)
	{4047-78	{460	{77	

* Quantum yield for blue light superimposed on red light, calculated for an irradiation period following a period in the dark.

† Quantum yield for red light superimposed on blue not determined.

the same rate, any change in the number of algal cells per unit volume of the suspension during an experiment occurs only at the top and bottom of the reaction vessel and not at, or near enough to, the cathode to have an effect on the rate of change in concentration of dissolved oxygen as determined by this method.

It is obviously necessary that respiration remain the same during the dark periods of an experiment, because in obtaining the rate of photosynthesis a correction must be applied which takes into account the oxygen consumed by the algal cells in the continuing process of respiration. There is apparently no way of avoiding this correction when quantum yields are determined from experiments using alternating light and dark periods. By selecting only those experiments in which the rate of respiration was constant and those which showed no appreciable change in rate during the dark period following a light period, it was felt that the calculated rate of photosynthesis most closely approached the true rate.

The generalization can be made that green cells low in food reserves do show an increased rate of respiration following a period of photosynthesis. Yet, if the rate of respiration of the green cells under a particular set of physiological conditions prevailing during an experiment of relatively short duration shows no change for the periods of darkness preceding and following exposure to light, and if the apparent photosynthesis is the same for a second light period immediately following the second dark period, the rate of respiration is apparently unaffected by the product, or products, of photosynthesis. That this situation has been very nearly attained in these experiments is indicated by the lack of change in the rate of respiration for successive dark periods as shown in Figure 11.3. Furthermore, any appreciable direct effect on respiration of light (8) of any one of the colors used should be detected in those instances in which the second determination of photosynthesis is made with light of a color differing from that used for the first determination, or in which one color is superimposed on another. No such effect was detected.

The quantum yields of photosynthesis obtained when the algal cells were irradiated with light of a single color are in very good agreement with the yields obtained in these laboratories for *Chlorella* (4, 5, 6) and for *Nitzschia closterium* (7). They are in good agreement with the yields obtained for *Chlorella* by Emerson and Lewis (8) who found the quantum efficiency of photosynthesis to be virtually the same for the red, green, and blue regions of the spectrum at low light intensities. They are lower than the yields reported by Warburg and Negelein (3), by Eichhoff (10), and by Warburg (11, 12). While it has already been indicated above that

the red light used in these experiments contained wave lengths of radiation not utilizable in chlorophyll photosynthesis, and it is to be expected that the calculated quantum yield for this color is less accurate than those for the other colors, the difference in the quantum yield for red light and for any other color is probably not significant.

The quantum yields obtained through irradiation of the algal cells with additional light of another color are in very good agreement with those obtained for any single color. Since these determinations were accomplished by using the rate of change in concentration of dissolved oxygen produced by a single color as a base, and determining the increased rate of change in the concentration of dissolved oxygen due to an additional color of light, it was unnecessary to use any value for the rate of respiration determined while the algal cells were in darkness. Thus, there was no abrupt change from respiration to respiration-photosynthesis, or vice versa, and there should have been less shifting of the equilibria of the various chemical reactions in the cells. While no direct proof is offered that the quantum yields obtained for the additional light are entirely free from errors due to changes in respiration during the two-color irradiation, the constancy of the rates of change of dissolved oxygen during these periods lends support to the view that the quantum yields so obtained are actually free from error.

SUMMARY

Quantum yields of photosynthesis in the alga *Chlorella* exposed to red, green, blue, and violet light were determined on the basis of oxygen produced, using the dropping mercury electrode. With alternating dark and light periods, and applying the usual correction for oxygen consumed in respiration during photosynthesis, it was found that the quantum yield is very nearly the same for each color of light and is in the range of 0.1–0.08 molecule of oxygen evolved per quantum absorbed. When the need for applying a correction for respiration is obviated by using the rate of photosynthesis-respiration in one color of light as a base and increasing the intensity of the radiation by using additional light of a different color, the quantum yield for the additional light was found to be independent of the color and the same as that determined through the use of alternating dark and light periods. Since the quantum yield was the same regardless of whether or not a correction for respiration had to be applied, it is considered very likely that no appreciable change in respiratory rate affecting oxygen exchange took place during exposure of the algal cells to light.

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12.

Quantum Efficiencies for Photosynthesis and Photoreduction in Green Plants¹

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DURING the past ten years there has existed a controversy as to whether the *maximum* quantum efficiency for photosynthesis in green plants is very nearly equal to one-fourth or whether it has a value less than half as great. The principal evidence for the higher value is contained in the classical work of Warburg and Negelein (1) published in 1923. In 1938 a group working at the University of Wisconsin (2, 3) published the results of extensive measurements, and the highest of their values were only about a fourth as great as those of Warburg and Negelein. At that time it was not clear, however, whether the discrepancy was to be attributed to errors of observation in one or both of the experiments, or whether it meant that Warburg and Negelein had been more successful than the Wisconsin group in bringing the algae into a state conducive to maximum efficiency. The nature of the Wisconsin results was known rather widely in advance of publication, and by 1938 experiments were well under way in other laboratories, as well as at Wisconsin, to discover the cause of the discrepancy.

In 1939 it was shown by the present author (4) that if the procedures introduced by Warburg and Negelein were followed closely, a value of efficiency comparable to theirs was obtained. It was observed also that for algae suspended in a carbonate-bicarbonate buffer the yield was about half as great as for cells in culture medium. This result was interpreted to mean that the

¹The experiments described in this article were performed in 1938-41; their publication has been delayed because of war work and subsequent distractions. The work was done in the Chemistry Department of the University of Chicago as part of the program directed by Professor James Franck and supported by the Fels Foundation. The Works Project Administration provided as an assistant Mr. Charles Burkhardt, to whom thanks are due for carrying out much of the routine work. Mrs. Clara Gaffron kindly assumed the responsibility for supplying cultures of algae.

buffer had an adverse physiological effect. It was concluded that the Warburg-Negelein procedure could conceivably conceal a systematic error, and that efforts should be made to improve the procedure. The weaknesses appeared to arise principally from the use of very dense suspensions of algae—the suspension is made dense enough so that substantially all of the incident light is absorbed. The photometry is thus simplified because the absorbed light may be taken as equal to the incident light.

On the other hand, the correction for respiration becomes relatively large. Since the greater part of the suspension is illuminated only feebly, it contributes little to the observed rate of photosynthesis but nevertheless contributes its full share to the respiration. Inasmuch as one wishes, in principal, to measure the upper limit to the quantum efficiency, and the efficiency tends to increase at lower light intensities, one makes the measurements at an intensity so low that a further reduction in intensity does not lead to any observable increase in efficiency.

Under these conditions, with the method of dense suspensions, photosynthesis merely compensates, or may even fail to compensate completely, the respiration that occurs simultaneously. That is to say, the correction for respiration equals or exceeds the true rate of photosynthesis. Consequently, the relative precision of the final result is inferior, or at best equal, to that of the correction for respiration. Moreover, since the respiration can be measured independently of photosynthesis only before or after the illumination, the constancy of the respiration is an important consideration. In order to minimize any change in the rate of respiration that might occur during the illumination period, Warburg and Negelein adopted ten-minute illumination periods. A possible objection to this procedure is that transient effects may falsify the results, for it is the quantum efficiency characteristic of steady-state conditions that should be measured.

It was concluded from this argument that it would be desirable to make measurements of quantum efficiency on suspensions of algae that would absorb only a moderate fraction—say less than one-half—of the incident light. The respiration would then enter into the final result with far less weight. Also, moderate variations in its rate would not vitiate measurements made with illumination periods sufficiently long to insure the establishment of steady-state conditions.

While the experiments were getting under way, new results on quantum efficiencies were published by the Wisconsin group (5, 6, 7) and by Emerson and Lewis (8, 9). On the basis of those results, the original program was modified considerably. For

instance, it had been intended initially that measurements employing the Warburg and Negelein procedure would be made in conjunction with those using the modified procedure. This was planned in order to exclude the possibility that conflicting results might be caused simply by differences in the condition of the algae. The plan was abandoned when it became evident, through the work of Emerson and Lewis, that because of peculiarities in the exchange of CO_2 by the algae, the original Warburg and Negelein procedure cannot be relied upon to yield a true quantum efficiency for photosynthesis (errors of the order of 100 per cent are possible) and therefore would not serve the intended purpose.

The later publications referred to were in agreement that the maximum efficiency is about 0.10 rather than 0.25. (While it is true that Eichhoff published at about the same time, results that accorded with the higher value, it is doubtful that much weight should be given to his result; this question is discussed at some length by Emerson and Lewis (9).) It may be stated in advance that the results to be reported here concur with the lower value.

Although the new results mentioned above appeared to settle the controversy in favor of the lower value, the importance of the quantity in question seemed to justify the completion of the measurements then in progress. Furthermore, the particular method used here appeared to have some special advantages. Also it could be readily adapted to the measurement of the quantum efficiency for the type of assimilation in which the consumption of hydrogen takes the place of the evolution of oxygen. This process (photoreduction) is known to occur in some species of purple bacteria and has also been found by Gaffron (11) to occur in some species of green plants after they have been given an anaerobic treatment with hydrogen. It seemed advantageous to measure the quantum efficiencies for both photoreduction and photosynthesis upon similar samples of plant material.

METHODS AND APPARATUS

In order to determine a value for the quantum efficiency, it is necessary to measure the amount of photosynthesis (expressed either as amount of CO_2 absorbed or amount of O_2 produced) and the amount of light energy absorbed by the plants. Alternatively, one may measure the corresponding rates. The determination thus consists of both a chemical and a photometric measurement.

In the present experiments, the photosynthesis was measured by means of a Warburg-Barcroft manometer. Inasmuch as detailed descriptions of the instrument and its use are readily available, they will not be repeated here.

The manometer was read by means of a double cathetometer which made it possible to take accurate readings without stopping the shaking of the manometer. In order to reduce the size of the field of illumination needed to cover the bottom of the flask at all times, the shaking excursion was limited to about 1 cm. With about 250 oscillations per minute, the agitation was sufficient so that 90 per cent of a change in the rate of pressure change occurred in five minutes.

All of the present quantum efficiency determinations are based on the measurements of rates. Manometer readings were made every two minutes and plotted against time, and the slopes of the curves were used in computing the rates. A rate of photosynthesis was based upon the last twenty minutes of a half-hour illumination period. Its respiration correction was determined from the second ten minutes of the dark period directly following the photosynthesis.

The rate of absorption of light energy is determined as the product of three measured quantities: the intensity of illumination, the cross-sectional area of the flask normal to the light beam, and the fractional absorption. The measurement of the first two quantities is quite straightforward. The third is more complicated because (a) the suspension of algae is a turbid medium and therefore scatters as well as absorbs light, and (b) the necessary agitation of the reaction vessel results in a non-uniform absorbing layer.

It can be shown that the absorption is not appreciably affected by the agitation of the flask, provided the average absorption is not greater than about 40 per cent. For estimating the possible error, it is sufficiently accurate to assume that for small absorptions the intensity falls off exponentially with distance, even in a turbid medium. Suppose that with the suspension uniformly distributed the absorption is 40 per cent. If now the depth is decreased by one-half over half of the flask and increased an equal amount over the other half, the absorptions are respectively $1-0.6^{3/2}$ and $1-0.6^{1/2}$, or 0.535 and 0.225. The average is 0.380, or a decrease of 5 per cent from the value for a uniform depth of suspension.

In the experiments, the shaking was so regulated that during only a fraction of the cycle were the irregularities in the depth of the suspension as great as that assumed above. Consequently, the time average of the absorption differs by, at most, a few per cent from that with a uniform depth.

The absorption values of the suspensions were measured by means of an integrating sphere, illustrated in Figure 12.1. A sphere has been used previously for the same purpose by Vermeulen, Wassink, and Reman (12). The sphere provides a rigorous method

for measuring the true absorption of light in a turbid medium, independent of the scattering.

In first approximation, the response of the photoelectric cell is directly proportional to the product $F(1-f)$, where F is the incident flux, the quantity $1-f$ is the fraction of light scattered

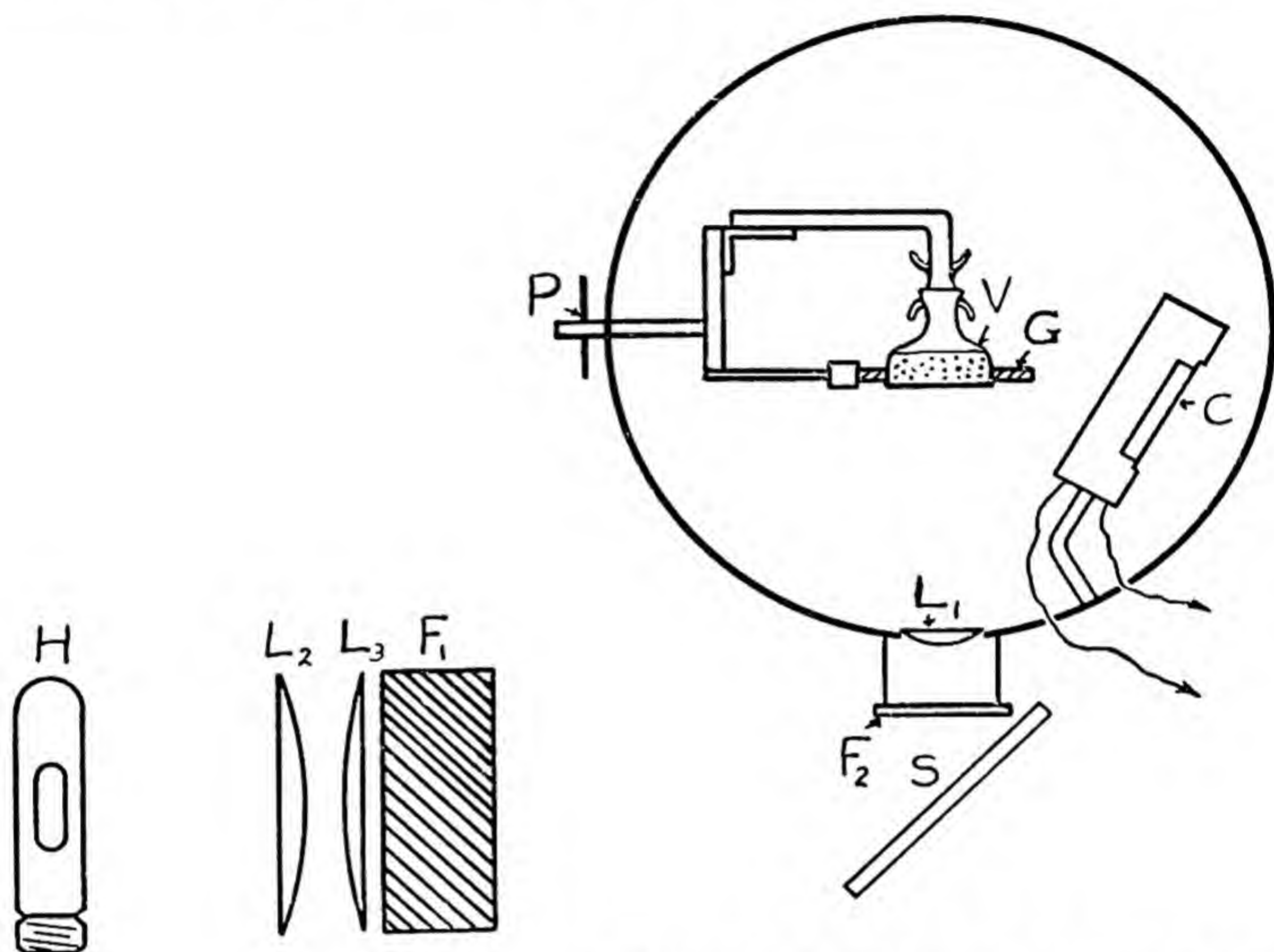


FIG. 12.1—Integrating Sphere. C, photocell, hooded so as to receive light only from an indirectly illuminated area of the sphere; G, opal glass; V, flask containing suspension; P, pivot, permitting either V or G to be swung into the light beam; L_1 , projection lens; F_2 , glass filter; S, mirror; F_1 , CuSO_4 filter; L_2 , L_3 , condenser lenses; H, mercury lamp.

and transmitted by the suspension, and f is the true absorption. An approximate value of absorption might be obtained by simply comparing the photoelectric currents observed with the flask in the light beam and out of the beam. However, the value so obtained would be subject to errors caused by imperfections in the sphere, non-uniform distribution of the scattered light, reflection from the lower surface of the flask, and reflections and scattering by the lens which focuses the light upon the flask.

These errors can be eliminated by making comparisons between the suspension of algae and two standards; (a) ink, for which

$f = 1$, and (b) a thin suspension of calcium carbonate, for which $f = 0$, made up to have a turbidity roughly equivalent to that of the suspension of algae. Actually, the suspension and each of the standards is compared with the opal glass shown in the illustration. Apparent transmissions for each are taken as the ratio (deflection with flask illuminated) / (deflection with opal glass illuminated). If x , y , and z are the respective apparent transmissions of the algae, calcium carbonate, and ink, then the true fractional absorption by the algae, for light actually entering the suspension, is given by

$$f = (y-x)/(y-z).$$

The absorption values as determined above are subject to a small correction. For convenience, the absorption measurements are made with the flask in air, although when the flask is in place on the manometer and in the constant temperature bath, it is in water. The walls of the flask reflect some of the scattered light back into the suspension, and the amount reflected by the outer surface is less in water than in air. This effect was measured in a series of trial experiments, with the result that absorption values between 0.20 and 0.50 must be multiplied by 0.98 to correct for it.

The optical arrangement for the photochemical part of the experiments is illustrated in Figure 12.2, and is for the most part self-explanatory. With large aperture condensers there is a tendency for the illumination to fall off at the edges. This tendency may be compensated by taking advantage of the aberrations of the thick lens L_1 . By shifting the position of the arc image, which acts effectively as a stop for this lens, it is possible to bring up the illumination at the edges of the field. A typical measurement of the intensity over seven areas, which together practically covered the region normally occupied by the flask, showed that the average intensity was 0.4 per cent greater than the intensity at the center. The RMS deviation of the seven measurements from their average was 3 per cent. This nonuniformity can introduce an error only as a second order effect in so far as the *time average* of the distribution of depth of the suspension is nonuniform.

The intensity of illumination was measured with the surface thermopile, which was calibrated against lamps from the U. S. Bureau of Standards. The calibration proved to be independent of whether the plane of the thermopile was vertical or horizontal. The optical bench that carried the illuminating system was mounted on rails so that the light beam could be transferred from the reaction vessel to the thermopile. Optical equivalence of p_1 , the midplane of the suspension, and p_2 , the plane of the thermopile, was insured by placing an object in contact with the condenser L_3 and measuring

its image in the two planes. Corrections for reflection losses had to be applied. For the thermopile there are two air-glass interfaces; for the suspension there are three air-glass and three water-glass interfaces. The transmission of the double glass bottom of the thermostat was checked by direct measurement. All measurements were made with the 578 m μ line of mercury isolated by filters.

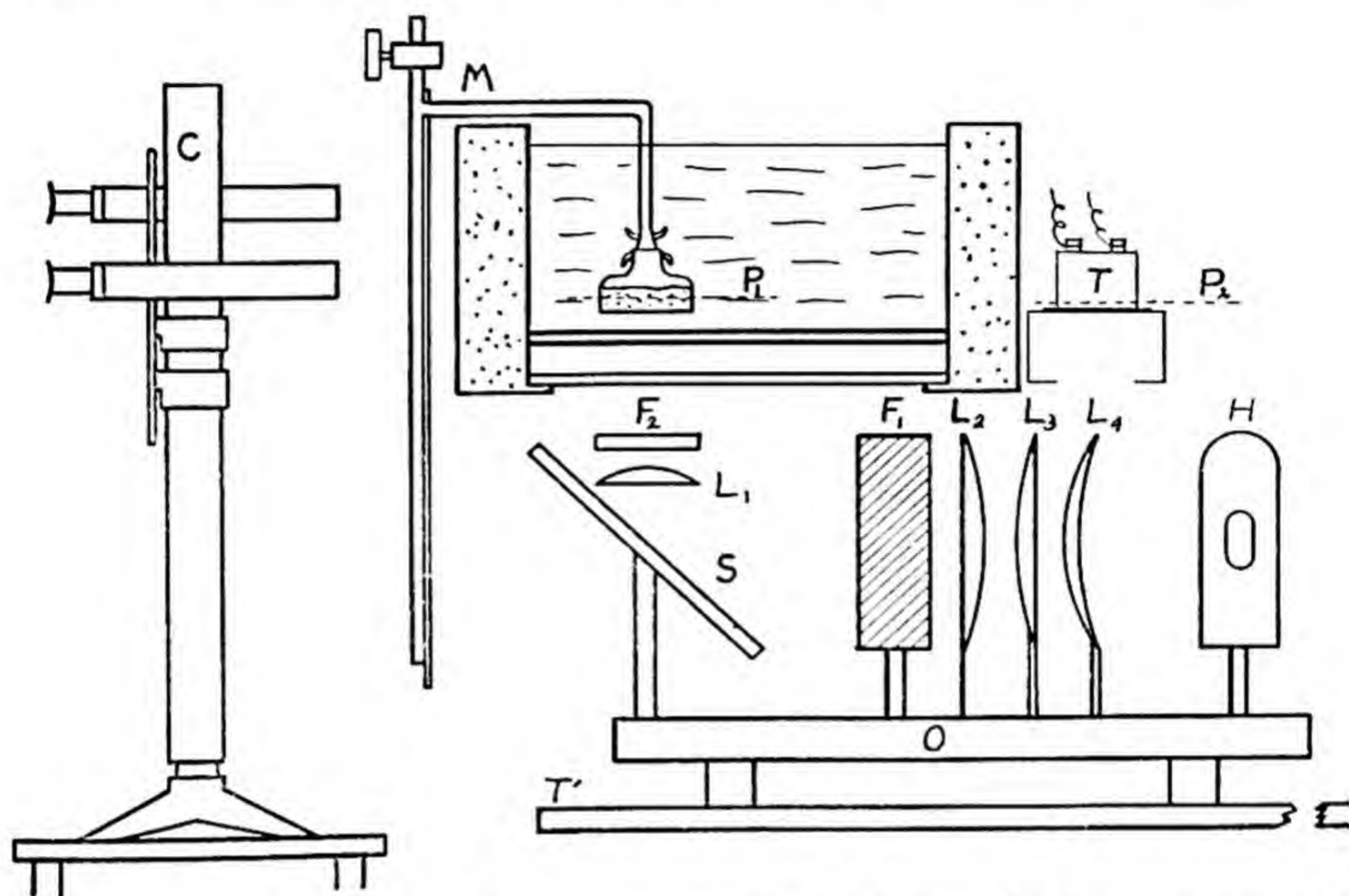


FIG. 12.2—Apparatus for Measuring Photosynthesis and Photoreduction. C, cathetometer; M, manometer; P_1 , midplane of suspension; T, thermopile; P_2 , plane of thermopile; H, mercury lamp; L_2 , L_3 , L_4 , condenser lenses; F_1 , CuSO_4 filter; S, mirror; L_1 , projection lens; F_2 , glass filter; O, optical bench; T' , track on which optical bench moves.

Measurements were made with two species of algae, *Chlorella pyrenoidosa* and *Scenedesmus* sp. Both were grown as pure cultures. Illumination for growth was provided by tungsten lamps. The culture medium for *Chlorella* was that described by Emerson; for *Scenedesmus*, a modified Benecke solution. During the growing period a slow stream of 4 per cent CO_2 in air was bubbled through the cultures and they were shaken continuously to prevent settling. *Chlorella* was illuminated continuously; *Scenedesmus* was given an occasional dark period of about half a day. In attempts to increase the quantum efficiency, cells were grown at various light intensities and in a few cases in modified culture media. Cultures were grown to various densities, and with this variation only did the quantum efficiencies show a clear-cut correlation.

MEASUREMENTS

PHOTOSYNTHESIS IN BUFFER SOLUTIONS

The method employing carbonate-bicarbonate buffers is the most convenient for measuring photosynthesis manometrically. This method has the advantages of being unambiguous—since the manometer responds only to the exchanges of oxygen—and is more sensitive than the other methods. These buffers have been very widely used in investigations of photosynthesis. They have been used in many of the present experiments, both to test the apparatus and method and to find how the quantum efficiency is related to culture conditions and other factors. The absolute value of the efficiency obtained with buffers may be open to some objection, however, because the buffers have a fairly low pH and may therefore not provide the most favorable physiological conditions. It should be remembered also that with buffers no information is obtained about the exchange of CO_2 .

As a test of the measurements of absorption, measurements were made with different quantities of cells from the same culture. It was found that the observed quantum efficiencies with partial absorption did not vary systematically with the absorption, provided the absorption was less than 50 per cent and the shaking was not too violent. It was also determined that there was no discrepancy between values observed with partial and with total absorption.

Employing the same suspension, identical values of quantum efficiency were observed at 10°C . and 20°C . In another experiment measurements were made with a buffer of composition $0.090\text{M KHCO}_3 + 0.010\text{M K}_2\text{CO}_3$. Enough carbonate was added to bring the composition to $0.085\text{M KHCO}_3 + 0.015\text{M K}_2\text{CO}_3$, thereby changing the CO_2 concentration in the ratio 8:5. There was no change in the observed quantum efficiency. In all subsequent experiments with buffers, the latter composition (Warburg's No. 9) was used.

In searching for the conditions most favorable to a high quantum efficiency, the following variations in culture were made with *Chlorella*: (a) Illumination during the last few days of growth was varied from 10,000 lux downward; (b) The concentration of salts in the culture medium was doubled; (c) An A-Z solution of microelements was added to the culture; (d) Cells were partially fractionated by centrifuging; (e) Normal air, instead of 4 per cent CO_2 in air, was supplied during growth; (f) Cultures were grown to different densities of cells. In general, it was found that the highest efficiencies were observed when the growth of the culture was not continued much above a density of one ml. packed wet cells per liter of nutrient solution. None of the other variations indicated had a clear-cut effect.

As part of the quantum efficiency determinations, light absorption and respiration measurements were made on cells from each culture. In addition, cell volumes and maximum rates of photosynthesis (using white light) were measured. The results may be summarized by saying that strong illumination during growth results in a high saturation rate of photosynthesis, a low chlorophyll content, and a high rate of respiration. These observations are in agreement with those made by Sargent (13).

TABLE 12.1

Experiments	Quantum Efficiency, ϕ , moles/einstein	Intensity ergs/cm. ² sec.	10°C. rate/max. rate	Intensity at Compensation ergs/cm. ² sec.	Culture Density cmm./ml.	Intensity for Growth lux
1.....	0.083 ₂ 0.086 ₂	4,400 1,900	0.23 0.10	500	0.2	very low
2.....	0.079 (0.094)	4,400 1,900	0.17 0.09	3,800	1.5	10,000
3.....	0.081 ₂ 0.084 ₂	4,400 1,900	0.15 0.06	2,600	1.1	10,000
4.....	0.078 0.081	4,400 1,900	0.23 0.10	1,100	3,000
5.....	0.077 ₂ 0.076 ₂ 0.077	4,400 2,100 1,800	0.24 0.11 0.10	500	3	2,000
6*.....	0.074 ₂ 0.082 ₂	4,400 1,900	0.13 0.06	1,700	1.5	10,000
7*.....	0.072 ₂ 0.079 0.077 ₂	4,400 1,900 1,100	0.18 0.08 0.04	500	2	2,000

* *Scenedesmus* (all others *Chlorella*).

The results of the last five experiments on *Chlorella*, which include those made under the more favorable conditions, are summarized in Table 12.1; two experiments on *Scenedesmus* are also included. The second column contains the observed quantum efficiencies. Values without subscripts are individual values; those with the subscript 2 are the averages for two periods of illumination. The third column indicates the intensity of illumination for the efficiency measurement. The fourth column shows the compensation

intensity for the culture, that is, the intensity (determined by interpolation) at which respiration and photosynthesis would just balance. The fifth column gives the ratio of the rate of photosynthesis for the efficiency measurement to the maximum rate for the same cells in strong light. The sixth column gives the volume of packed wet cells per unit volume of nutrient. The seventh column shows the intensity, in lux, at which the last two days of growth took place.

Table 12.1 contains only one value of ϕ that exceeds 0.086 (the one in parentheses). This value is judged to be unreliable because it represents only one measurement. A second measurement on the same suspension at intensity 4,400 indicated that the culture was not superior to others. Inasmuch as it seems likely that this isolated high value has been influenced by some error in measurement, it should not be regarded as significant in judging the upper limit to the quantum efficiency. The remaining experiments show only a small difference between the quantum efficiencies observed at intensities 1,900 and 4,400 in that the former average 5 per cent higher for *Chlorella*, 8 per cent for *Scenedesmus*.

One may conclude that at intensity 1,900 photosynthesis of these algae is within the light-limiting region of intensities. The upper limit to the observed values is thus 0.086; the random error judged from comparison of individual values is about 2 per cent. The possible systematic errors in the various factors entering into the measurements are estimated to be as follows:

incident flux	—	(transmission of tank and water, effective area
± 5 per cent		of bottom of flask, convergence of light beam,
		location of midplane of suspension)
absorption	—	(effect of shaking, absorption measurement,
± 3 per cent		spectral impurities)
manometer calibration		
± 1 per cent		

From these experiments, we may conclude that the maximum quantum efficiency for photosynthesis, as measured by the production of O_2 by *Chlorella* suspended in carbonate-bicarbonate buffer, is 0.086, with a possible error of about 11 per cent, or on the basis of probable error, 0.086 ± 0.05 .

Essentially the same value applies also to *Scenedesmus*. This value is about 20 per cent lower than that estimated from tabulated values for the same quantity given by Emerson and Lewis (9). Although this is a reproducible value, and the use of buffer provides a convenient method of making routine measurements for comparison purposes, the experiments described in the next section

indicate that somewhat higher efficiencies can be obtained in other suspension media.

SIMULTANEOUS MEASUREMENTS OF O_2 AND CO_2

A few additional measurements of the quantum efficiency for normal photosynthesis were made in connection with the work on photoreduction. In these measurements the algae, *Scenedesmus* sp., were suspended in 1/40M $KHCO_3$ solution saturated with 4 per cent CO_2 ; the gas exchange was computed with an assumed value of 1.0 for the assimilatory quotient. (Buffers are unsuitable for photoreduction; the algae were simply switched over, by strong illumination, to normal photosynthesis at the end of a run on photoreduction.) The values obtained in this way (0.103, 0.116, and 0.094) are appreciably higher than those observed with cells suspended in carbonate-bicarbonate buffers. Naturally, the magnitude of these values raised the question whether the efficiencies observed in buffer fall somewhat short of the maximum possible value because of some unfavorable physiological property of the buffer, or whether these higher values are in error simply because the assumed value of 1.0 for the assimilatory quotient is incorrect.

The occurrence of the high values cannot be attributed to the previous anaerobic treatment of the algae. A check experiment in which cells from the same culture were measured both with buffer and with the bicarbonate solution, without any anaerobic treatment, showed that in the latter case the efficiencies were consistently about 25 per cent greater. Nor does it seem that the "outburst of CO_2 " observed by Emerson and Lewis and found by them to be especially troublesome with *Chlorella*, is an important factor in these observations. After the normal five-minute adjustment period the manometer reading changed in a strictly regular fashion in both the light and the dark periods.

As a means of settling the question raised above, an experiment based on the principle of unequal volumes of suspension in the flask was undertaken in order that the exchanges of both O_2 and CO_2 could be measured directly, without reliance on an assumed value for the assimilatory quotient. The procedure for this experiment was similar to that employed by Emerson and Lewis (9). Two runs were made, similar in all respects except that in one case the cells were suspended in 20 ml. of bicarbonate solution, in the other in 5 ml. of solution. Emerson and Lewis used equal volumes of suspension, but employed flasks of different volumes to equalize the time-lags of the manometer for the two runs so that rapid changes in the rate of gas exchange would not be falsified. This consideration is not important in the present experiments,

which are based upon steady rates of exchange observed over periods long in comparison with the adjustment period of the manometer. Comparison measurements with buffer, using cells from the same culture, were also made.

Three similar suspensions of *Scenedesmus* were prepared in the following way. A culture that had been stored in the refrigerator for a few days was grown for one day at 7,500 lux and for an additional half day at 1,000 lux. About 130 ml. of the culture was centrifuged and the cells were taken up in bicarbonate solution. This suspension was then centrifuged slowly for a few minutes to remove the larger cells, as had been done for the photoreduction measurements. It was then divided into three equal parts and put in the refrigerator to be used as needed during the experiment, which started about ten hours later. The three samples were used respectively for measurement with 20 ml. of bicarbonate solution plus one per cent CO_2 in air, with 5 ml. of the same solution, and with buffer. At the conclusion of these measurements the remainder of the culture was taken from the growing bath and a fresh suspension prepared for another measurement with buffer, to find whether storage in the refrigerator had caused any gradual change in the quantum efficiency of the first three samples. The results are summarized in Table 12.2. The second and third columns give the observed quantum efficiencies and the fourth column the assimilatory quotient ($-\text{O}_2/\text{CO}_2$) computed from them. The fifth column gives the respiratory quotient ($-\text{CO}_2/\text{O}_2$) observed for the dark periods.

A similar experiment was performed with *Chlorella* suspended in culture medium. This was an earlier experiment and suffered some deficiencies in technique that were remedied in the experiment described above. The values observed were $\phi_{\text{O}_2} = 0.071$, $\phi_{\text{CO}_2} = 0.061$, $AQ = 1.21$, $RQ = 1.16$; and for the second determination, 0.085, 0.082, 1.23, and 1.03. With buffer, the values were $\phi_{\text{O}_2} = 0.075$ and 0.076. Although the accuracy of the results is somewhat uncertain, the discrepancy between the first and second measurements appears to be genuine. The results support in a qualitative way the conclusions drawn from the experiment with *Scenedesmus*.

From the above results one may conclude that the quantum efficiency for *Scenedesmus* is appreciably greater for cells suspended in 1/40M KHCO_3 solution than for cells suspended in the carbonate-bicarbonate buffer—the average values of ϕ_{O_2} from Table 12.2 are respectively 0.093 and 0.070. There is some doubt, however, that the value 0.093 taken from Table 12.2 represents the maximum efficiency possible, for the value 0.070 obtained in

buffer is somewhat smaller than the maximum value 0.082 given in Table 12.1 for a different culture of *Scenedesmus* also measured in buffer but at considerably smaller light intensities. Perhaps, also, the culture upon which Table 12.2 is based was somewhat inferior. It seems reasonable that a better estimate of the maximum efficiency can be obtained by multiplying the value 0.093 by the correction factor $0.082/0.070$. It would have been preferable to circumvent this correction by making more experiments of the type just described,

TABLE 12.2

QUANTUM EFFICIENCY FOR PHOTOSYNTHESIS, *SCENEDESMUS*, IN 1 PER CENT CO_2 IN $1/40\text{M KHCO}_3$ AND IN BUFFER, 20°C ., 5,300 ERGS/ CM^2 SEC.

	ϕ_{O_2}	ϕ_{CO_2}	A. Q. $-\text{O}_2/\text{CO}_2$	R. Q. $-\text{CO}_2/\text{O}_2$
1.....	0.091	0.075	0.95	1.21
2.....	0.095	0.081	0.93	1.18
3.....	0.069	In buffer		
4.....	0.071			
5.....	0.070			
6.....	0.068			

but that was impossible because the experiments had to be discontinued at that point. One thus obtains for the maximum efficiency of photosynthesis, based on the production of oxygen, the value 0.108; the probable error is estimated to be of the order of ± 10 per cent.

THE QUANTUM EFFICIENCY FOR PHOTOREDUCTION

Scenedesmus was selected as the plant most suitable for the experiments on photoreduction. When this alga, among others, is treated for several hours with hydrogen in the strict absence of oxygen, it becomes "adapted" and upon illumination absorbs CO_2 and H_2 simultaneously in the ratio 1:2. This process can be continued for a long period of time, provided the illumination is not too strong. If a certain limit of illumination is exceeded, the cells switch over to normal photosynthesis and produce oxygen. This limiting intensity for photoreduction cannot be located very definitely, inasmuch as the time for switch-over can vary from twenty minutes to one minute, according to whether the limit is only slightly or greatly exceeded. The switch-over is irreversible to the extent that, when it has occurred, the cells will not again absorb

hydrogen photochemically until the adaptation process has been repeated.

Photoreduction, as a function of light intensity, does not follow the saturation curve of normal photosynthesis but rather an S-shaped curve. The curves are similar to those found by French (14) for the same process in purple bacteria, but with the bacteria the S-shape is more pronounced. For comparison, the rate-intensity

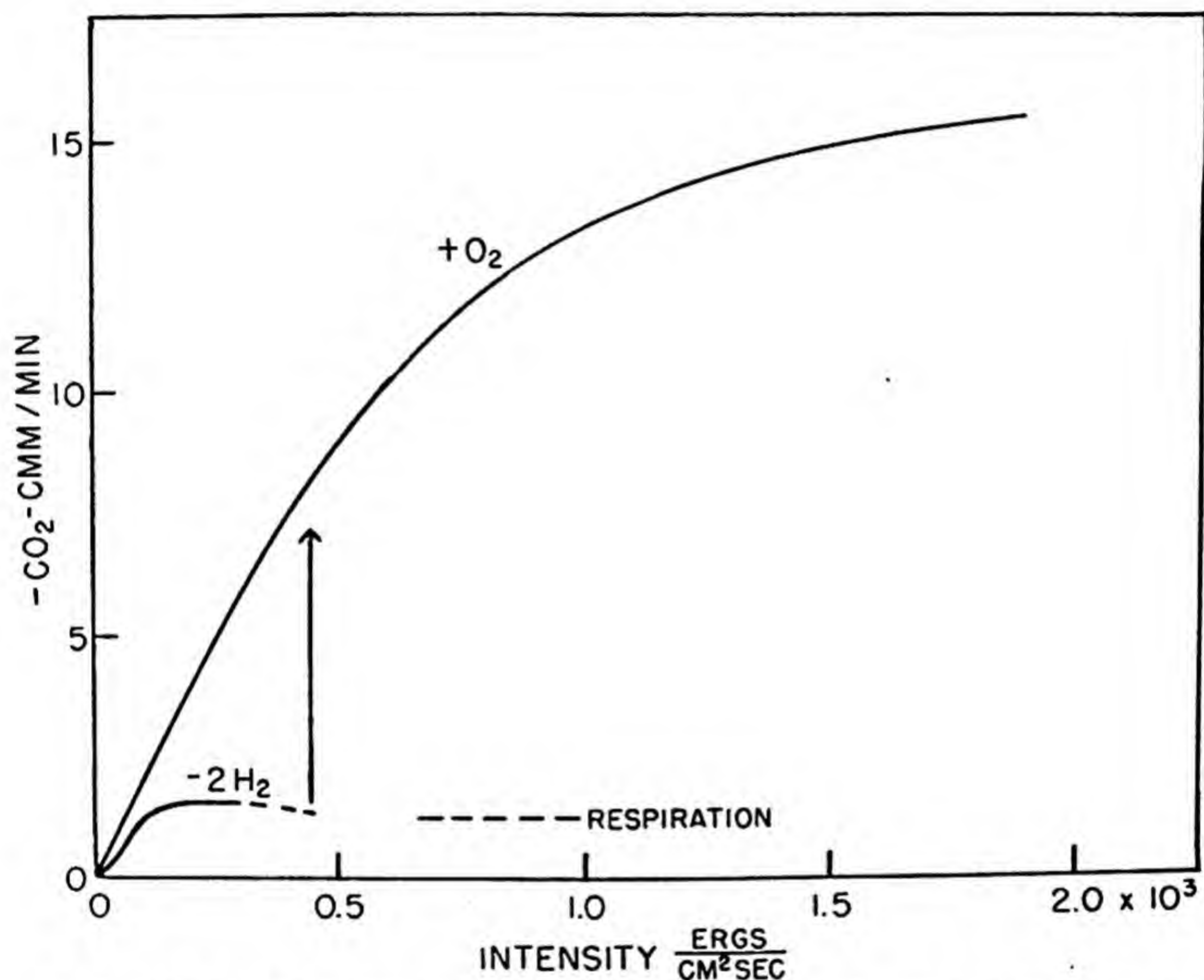


FIG. 12.3—Rate-Intensity Curves for Photosynthesis and Photoreduction. *Scenedesmus*, 44 cmm. cells, 20°C., 1/40 M KHCO_3 , 4% CO_2 + 96% H_2 . (The curve for photosynthesis is based on an assumed quotient of unity and is probably 10-20 per cent too high.) The dashed line shows the magnitude of respiration after photosynthesis.

curves for both photoreduction and photosynthesis are shown in Figure 12.3; the photoreduction curve is shown on a larger scale in Figure 12.4.

The maximum rates of photoreduction for the algae used in the efficiency measurements were from two to three volumes of CO_2 per hour per unit volume of wet cells. The magnitude of these rates is comparable to that of normal respiration rather than that of photosynthesis. Quantitatively the phenomenon tends to be slightly erratic. If two observations, made at one definite intensity,

are separated by a dark period or by a period at a different intensity, the rates may differ by 10 or 20 per cent. These differences are much too great to be attributed to any error of measurement. After a period of moderately intense illumination there is a dark reaction that has the same direction as the rate during illumination and, roughly, 5–10 per cent of its magnitude. It decays very slowly with

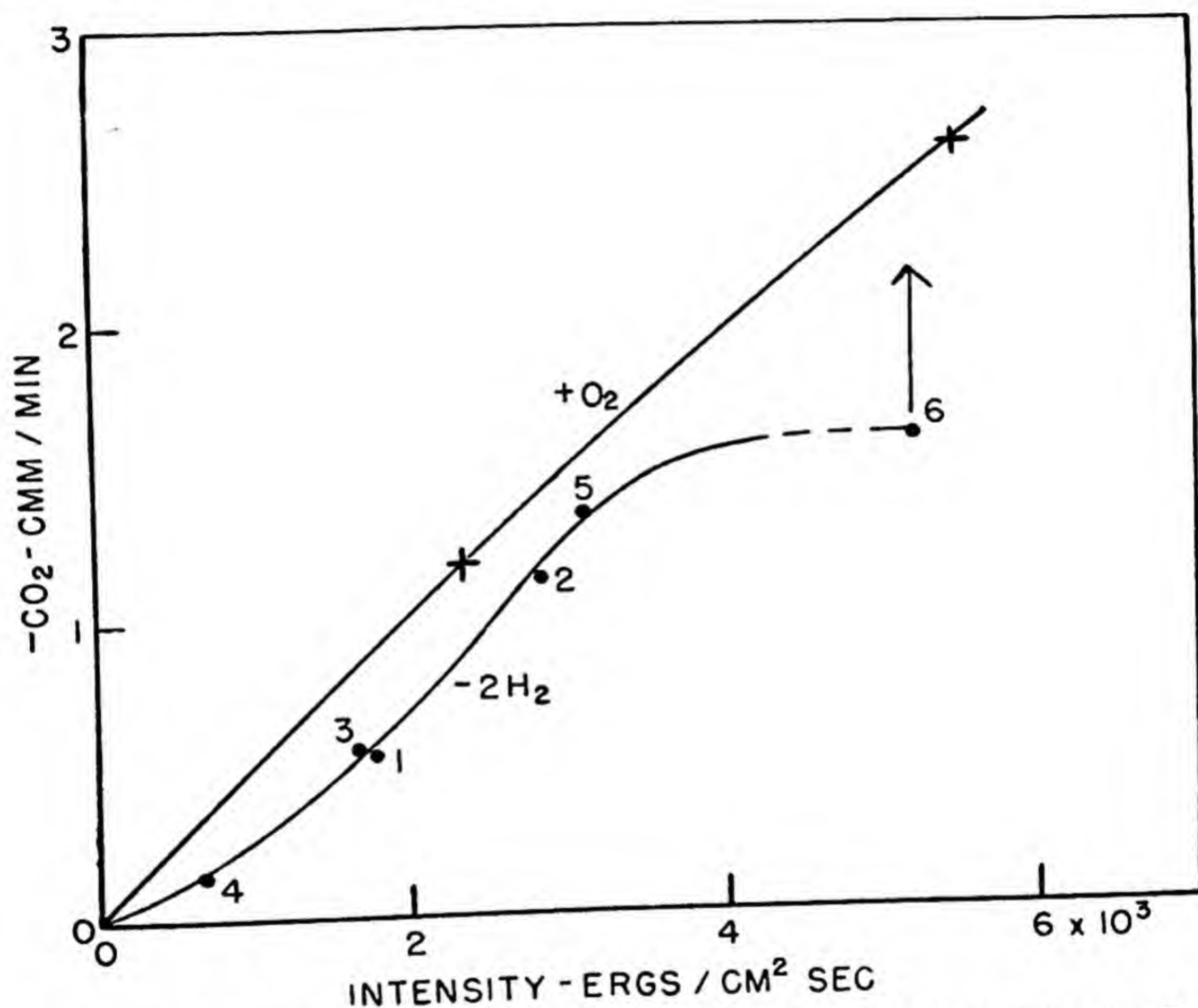


FIG. 12.4—Rate-Intensity Curves for Photosynthesis and Photoreduction. The same data as for Figure 12.3 but on a different scale. The numbers along the lower curve show the order in which the values were observed.

time. After illumination strong enough to produce saturation, the dark rate sometimes starts by being comparable to the preceding rate of photoreduction, falls to perhaps a third of its initial value in ten minutes, and then slowly continues to decay.

It is not possible to use carbonate-bicarbonate buffers in experiments on photoreduction because the algae become inactive after several hours of anaerobic treatment in these solutions. Consequently, it is necessary to suspend the cells in a solution that exchanges CO_2 freely. The two-vessel method is of doubtful value

in this case, where the exchange of H_2 has the same sign as that of CO_2 , is about twice as rapid, and the rates are not precisely reproducible. Accordingly, one is forced to depend upon measurements made with one vessel and upon a quotient determined by other means. Experiments performed by Gaffron (15) indicate that the quotient (H_2 absorbed)/(CO_2 absorbed) is, within a few per cent, equal to 2.00. This value was used in computing the constant of the manometer. The quantum efficiencies are expressed in terms of CO_2 absorbed.

There may be, in principle at least, some question as to whether Gaffron's value of the quotient holds under the conditions of the quantum efficiency measurements. Therefore, it is necessary to consider to how great an extent departures of the constant from the assumed value may affect the validity of the results. When the manometer flask is half-filled with water, about one-fifth of the pressure change is caused by the absorption of CO_2 and four-fifths by the absorption of H_2 , provided the quotient is 2.00. Computation shows that a 10 per cent departure of the quotient from the value assumed will lead to errors of about +2 per cent in the computed H_2 exchange and -8 per cent in the CO_2 exchange. Consequently, the quantum efficiencies as computed represent quite accurately twice the efficiency for the absorption of hydrogen, regardless of fairly large departures of the quotient from the assumed value. The uncertainty in the CO_2 exchange is about equal to that of the quotient. The results have been expressed in terms of CO_2 , however, in order to make them directly comparable to the values for normal photosynthesis.

The rate of photoreduction was taken as the difference between the rate during illumination and the rate for the following dark period. The justification for this correction is as follows. In the range of intensities where the quantum efficiency is a maximum, the transition at the end of an illumination period is rapid, and after the transition the dark rate changes slowly with time. The most plausible interpretation of this behavior is that the transition is a short aftereffect of the light—a genuine lag in the chemical kinetics of the photoreduction—and that the slowly changing process is a consequence of photoreduction, not a part of it. This correction can not be said to be as well justified as the corresponding correction for respiration in the case of normal photosynthesis, but it seemed preferable to make the correction for the dark reaction rather than to ignore it. Without the correction, the quantum efficiencies would be about 10 per cent greater. The shape of the rate-intensity curve is little affected by the correction.

In the computation of quantum efficiencies the corrected rate in the light, expressed in moles/sec., was divided by the absorbed light flux expressed in einsteins/sec., just as in the case of normal photosynthesis. Values of efficiency about 20 per cent higher than those given would have been obtained had they been computed from the maximum slope of the rate-intensity curve, as was done by French (14) in his study of purple bacteria. However, this procedure implies special assumptions regarding the cause of the knee in the curve at very low light intensities. In the absence of any convincing argument for using this method of computation in the present case, the author feels it is unjustifiable to depart from the conventional definition of the quantum efficiency. Although, as has been stated, the response of the manometer is governed almost entirely by the exchange of hydrogen, the efficiencies have been expressed in terms of CO_2 , taking the ratio $\text{CO}_2/\text{H}_2 = 1/2$, in order to make them directly comparable to the values for normal photosynthesis.

Except for the following details, the apparatus and procedure for the photoreduction measurements were the same as those described for normal photosynthesis. The algae were centrifuged from the culture medium and put into 1/40M KHCO_3 solution. The light absorption was measured, the flask was attached to the manometer and placed in the temperature bath; then 4% CO_2 + 96% H_2 was flushed through several times. The shaking of the manometer was started and the cells were kept in the dark for about ten hours (overnight); after this treatment the cells were "adapted" and the measurements could proceed.

The routine for the measurement of the quantum efficiency was the same as that described for normal photosynthesis. Measurements at various light intensities were made, and in some experiments the illumination was increased until the cells switched over to oxygen production. After the rate of photosynthesis had become constant, two or three measurements of the quantum efficiency for normal photosynthesis were made. The flask was then removed from the manometer and the absorption remeasured; in no case did it differ from its value at the start of the experiment, at least eighteen hours earlier, by more than 5 per cent.

In one experiment the mixture 4% CO_2 + 96% H_2 was used for several measurements and then replaced by 4% CO_2 + 48% H_2 + 48% N_2 ; the quantum efficiency was very little altered by this change. This observation shows that the rate of the reaction is not limited by the H_2 concentration corresponding to a partial pressure of one atmosphere. As part of the same experiment, addi-

tional measurements were made on the same suspension on succeeding days. It was found that only on the third day had the quantum efficiency decreased to one-half of its initial value. This observation shows that *Scenedesmus* suspended in 1/40M KHCO_3 solution is not harmed appreciably by twenty-four hours of anaerobic treatment.

In the earlier experiments on photoreduction the cells were simply centrifuged from the culture medium and put into bicarbonate solution. The quantum efficiencies for photoreduction were then about half as great as the values observed subsequently for normal photosynthesis. It was found later that the efficiency for photoreduction was much higher if the centrifuging was done in such a way as to select small cells. A portion of the culture was centrifuged until the medium was just perceptibly green. The packed cells were then taken up in bicarbonate solution and centrifuged until three-quarters or more of the cells had settled. The settled cells were discarded and those that remained in suspension were used for the experiment. In eight experiments with cells prepared in this way, only one gave a low quantum efficiency.

The results of the seven experiments that yielded high efficiencies are plotted in Figure 12.5. Perhaps at first sight the most prominent feature of this figure is the scatter of the points, but this characteristic is less disturbing if attention is focused on the results of an individual experiment. Even then, however, there remain discrepancies of the order of 10 per cent between points measured at nearly equal light intensities. These discrepancies are several times greater than those that occur in the measurement of normal photosynthesis and therefore cannot be ascribed to uncertainties in the physical measurements. Apparently the quantitative aspects of the course of the photoreduction process depend to a perceptible degree upon details of the recent past history of the cells, but the relation is not at all obvious. Actually, the effect is relatively small and would seem in itself to introduce an uncertainty of perhaps 10 per cent in the estimated upper limit of the observed quantum efficiencies.

A second feature readily discernible in Figure 12.5 is the tendency of the higher values to occur at intermediate light intensities. This characteristic corresponds to the S-shape of the intensity-rate curves.

From the data presented in Figure 12.5 and from the discussion of errors, the maximum quantum efficiency for photoreduction in *Scenedesmus* is estimated to be 0.110 ± 0.015 . It should be pointed out that some arbitrariness enters into this estimate because of peculiarities in the photoreduction process that are not understood. It has been assumed above that the correction for the dark rate

has been applied properly and that no correction need be made for the S-shape of the intensity-rate curve. By manipulating these corrections—in ways which at present seem to be entirely unjustified—the data might be reconciled with any value within the limits 0.090–0.160, but these are the extreme limits. The first limits

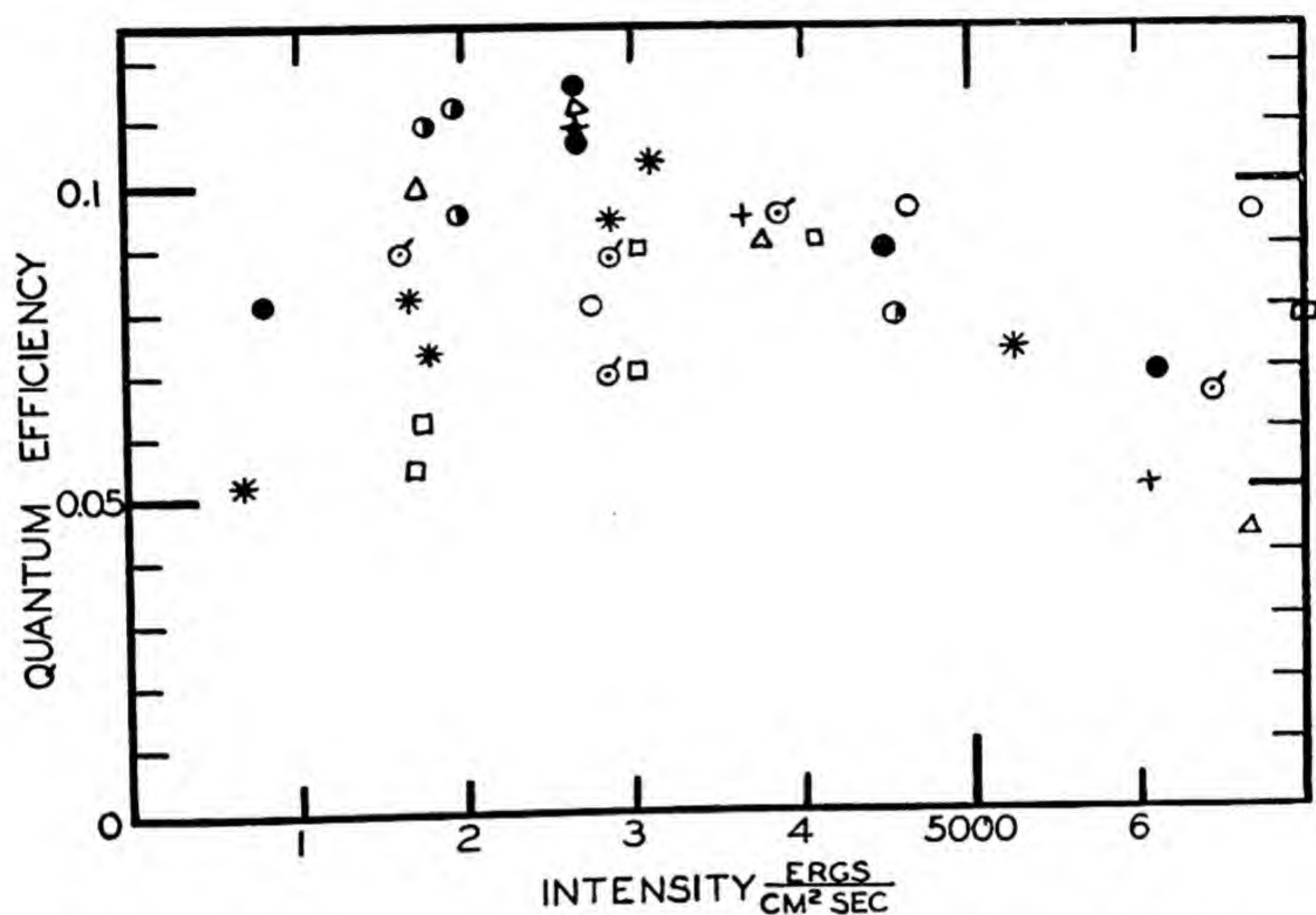


FIG. 12.5—Quantum Efficiencies for Photoreduction. The experiments are distinguished from one another by the various symbols. All data except + are for 4% CO₂ + 96% H₂. The + are data for 4% CO₂ + 48% H₂ + 48% N₂, taken on the same suspension as the ●, but after the gas mixture had been changed. In a few experiments photosynthesis was observed after a switch-over and the quantum efficiencies were computed on the basis of an assumed quotient of unity. These values are subject to perhaps as much as 20 per cent error, but they suffice to show that the cells were in good working order at the end of the photoreduction measurements. Observed values for photosynthesis are: ● 0.103, *0.116, and △0.094.

given (0.095–0.125) are regarded as the more reasonable interpretation of the data. Within the limits of error, the value derived above is identical with the efficiency for normal photosynthesis.

DISCUSSION

To the question of whether the maximum quantum efficiency of photosynthesis is 1/4 or “about 1/10,” the value 0.11 ± 0.01 obtained from the present measurements definitely speaks for the lower value. To arrive at a “most probable” value for the

efficiency, taking into account and evaluating critically all of the published measurements, would in itself constitute a large problem and cannot be undertaken here. An extensive bibliography is contained in the paper by Emerson and Nishimura above, and the subject is to be reviewed in the forthcoming second volume of Rabinowitch's treatise on photosynthesis. In the opinion of the author, the present situation can be summarized by saying that a preponderance of evidence indicates that the maximum efficiency lies between the limits 0.09 and 0.11, and that there is no unequivocal evidence that it is appreciably greater than 0.12.

While it is true that two recently published values exceed 0.12, they both are open to some objection. Kok (16) obtained the value 0.13, but it seems very likely that his result is as much as 10 per cent too high because of a lapse in photometry. Kok's measurements are based on essentially the same method as that used in the present measurements. He used thin suspensions of algae and determined their light absorption with an integrating sphere. The weak point in his procedure consists in measuring the absorption with a collimated beam of light and then using an extended source for the illumination during photosynthesis. The suspensions are contained in a flat shallow flask, and in the measurement of absorption the unscattered light has a path through the suspension equal to its depth. With the extended source, however, the path is on the average greater than the depth of the suspension. Consequently, the effective absorption for photosynthesis is greater than the measured value, so the flux actually absorbed is greater than that calculated. There is a small additional error of the same sign because of the fact that with an extended source some light enters the suspension through the sides of the flask.

Warburg (17) has recently described experiments that he believes substantiate his former value 0.25. However, according to experiments by Emerson (18) it is not certain that these new measurements are uninfluenced by an anomaly in the exchange of CO_2 . Warburg's measurement of the quotient, upon which the computation of efficiency depends, was not made on the same suspension of cells that was used in the efficiency measurements.

The observation that the maximum quantum efficiency for photoreduction is the same as that for normal photosynthesis lends support to the idea that photoreduction proceeds through the very same sequence of primary photochemical reactions as does normal photosynthesis. Other experiments have shown that the *flash saturation* and the *time for recovery between flashes* for photoreduction are also equal to the corresponding values for normal photosynthesis (19). Considered jointly, the above observations

lend added weight to the inference—previously drawn from evidence of an entirely different nature—that the process of adaptation modifies only an accessory part of the complex photosynthetic mechanism, while it leaves the remainder of the mechanism intact in organization and function (20). According to these interpretations, the adaptation process alters the enzymatic reactions that dispose of the oxidizing product formed concomitantly with the reduction of CO_2 . In the case of photosynthesis the oxidizing product oxidizes water to O_2 , while in photoreduction it oxidizes H_2 to water.

In conclusion, it may be of some value to consider the problem of further increasing the precision with which the maximum quantum efficiency of photosynthesis is known. In this discussion it will be taken for granted that the present limits of uncertainty are approximately those estimated above, namely 0.09–0.11. Presumably a more accurate result would be of interest largely as a means of deducing the number of photochemical steps contained in the reaction-scheme of photosynthesis. It seems doubtful, however, that efficiency measurements in themselves can ever reveal this number with complete certainty, for one knows only that the maximum efficiency is equal to *or less than* the reciprocal of the number of photochemical steps.

Many factors tend to make the above relation an inequality rather than an equality; for example, fluorescence (actually negligible), absorption of light by pigments inactive photosynthetically, competing reactions such as photooxidations, and back reactions. The maximum efficiency is observed under conditions such that the combined effect of all the above factors is reduced to a minimum, but there is no *a priori* reason to suppose that it can be made negligibly small. Actually, the contrary seems more likely.

A further uncertainty is connected with the assimilatory quotient. Inasmuch as the quotient can depart appreciably from unity, it would seem to be pointless to expend effort on improving the measurement of oxygen unless one has sound justification for considering the O_2 production, rather than the CO_2 consumption, to be the more significant quantity. In the absence of such justification, simultaneous measurements of both O_2 and CO_2 should be made.

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13.

A Calorimetric Determination of the Quantum Yield in Photosynthesis

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THIS STUDY was carried out at the University of California and the Hopkins Marine Station during the years 1936 and 1937. It is being published because of the urging of Dr. Gaffron and because Warburg has re-opened the question of quantum yield in photosynthesis. The suggestion that the heating of a leaf when carrying on photosynthesis, compared to when it was not, could be used as a measure of photosynthesis, was first made by Dr. Spoehr in 1926 (1). Since that time there have been three publications (2, 3, 4) on the method.

The apparatus used in this study was a modification of Callendar's "Cup radio balance" (5), originally devised to measure the heat production of radioactive substances. Figure 13.1 shows a cross-section, to scale, of the apparatus used. The small silver cup is held in place by a network of thermocouples whose other ends are on the copper ring shown in the diagram. In addition to these thermocouples, used to measure the temperature difference between the silver cup and the aluminum block, there is one other thermocouple through which a measured electric current can be passed in either direction by means of a reversing switch. By using the Peltier effect the small cup can be either heated or cooled; thus the calorimeter can be used as a null instrument. One can determine the current that will provide exactly sufficient cooling to give zero galvanometer deflection.

Because of the small size, the period of the whole apparatus was small. The complete measurement of the heating due to a beam of light could be made in one to five minutes.

The light source consisted of a neon arc, filtered through a copper sulfate solution and a red filter, so as to give some ten or fifteen neon lines at about 6,600 Å. The cell material used was

suspended in either Knop's solution or one of Warburg's carbonate buffers and placed in the silver cup. The quartz lid on the cup was greased in place with stopcock grease to make sure that there was no cooling of the cup due to evaporation. The amount of cell material used varied from .05 to 4 mm.³ in different fillings. After the heat production of the photosynthesizing cells had been measured, the top part of the calorimeter was taken off and the cells were exposed to ultraviolet light which brings the process of photosynthesis to a stop.

A second determination of heat production was now made, giving us the total energy being delivered to the cup by the light beam. The method of calculating the quantum yield was as follows: Let

H_0 = the rate of heating of the cup, by the light beam, when the cells are not doing photosynthesis (after the ultraviolet light);

H = the rate of heating of the cup when the cells are doing photosynthesis.

Both H_0 and H are measured as the heat production over and above that due to respiration. The assumption is implicitly made that the light does not change the rate of respiration. Further, let

Q = the rate at which light energy is being converted to potential energy by the process of photosynthesis;

F = the efficiency of photosynthesis;

Γ = the number of light quanta absorbed per CO_2 molecule reduced.

Since

$$Q = H_0 - H \quad (1)$$

and

$$F = \frac{Q}{H_0}, \quad (2)$$

we see that

$$F = \frac{H_0 - H}{H_0}. \quad (3)$$

We also know that

$$F = \frac{\Delta H}{\Gamma h\nu N} = \frac{112,000 \times 4.185 \times 10^7}{\Gamma \times 6.06 \times 10^{23} \times 6.6 \times 10^{-27} \times 4.55 \times 10^{14}} \quad (4)$$

when

ΔH = 112,000 cal. per mol.

N = Avogadro's number;

so that

$$\Gamma = \frac{2.57}{F} = \frac{257}{F\%}.$$

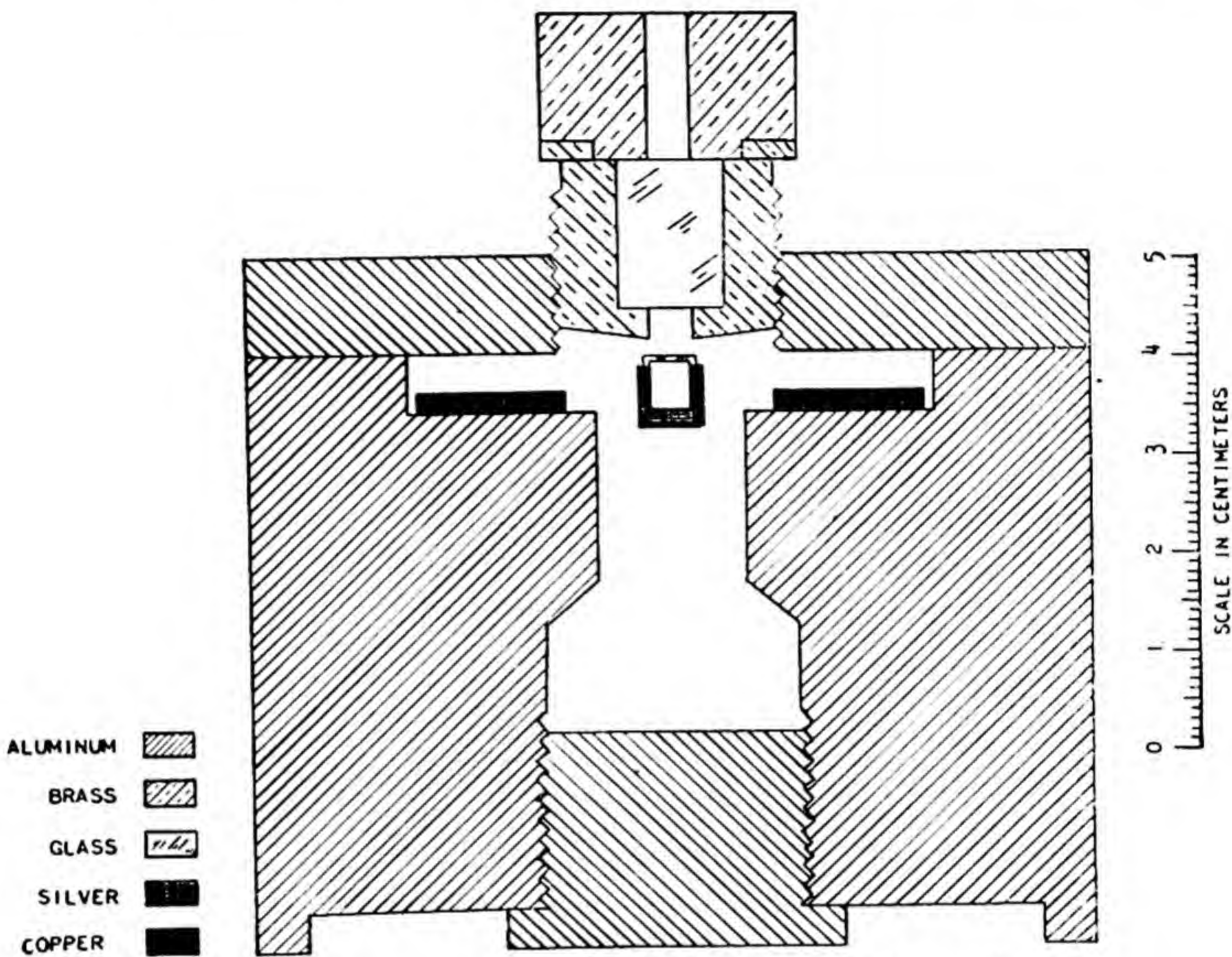


FIG. 13.1—Reaction chamber. See text for description.

TABLE 13.1
QUANTUM EFFICIENCY IN PHOTOSYNTHESIS. Γ = QUANTA PER MOLECULE
CARBON DIOXIDE

	H_o μ Watts	H μ Watts	Q μ Watts	F %	$\frac{\Gamma}{257} \cdot \frac{1}{F\%}$
<i>Chlorella pyrenoidosa</i>	5.08	3.70	1.38	27.2	9.5
<i>Chlorella pyrenoidosa</i>	4.60	3.80	.80	17.4	14.8
<i>Chlorella pyrenoidosa</i>	4.46	3.90	.56	12.5	20.6
<i>Chlorella pyrenoidosa</i>	2.24	1.90	.34	15.2	16.9
<i>Chlorella pyrenoidosa</i>786	.656	.130	16.5	15.6
Avocado Leaf912	.700	.212	23.2	11.1
<i>Chlorella vulgaris</i>	1.86	1.34	.52	27.9	9.2
<i>Chlorella pyrenoidosa</i>	16.2	12.9	3.3	20.4	12.6
Heat Production in Arbitrary Units					
<i>Scenedesmus</i> sp.	2.63	2.04	.59	22.4	11.5
<i>Scenedesmus</i> sp.	3.24	2.40	.84	25.9	10.3
<i>Chlorella pyrenoidosa</i>	2.08	1.65	.43	20.7	12.4
<i>Chlorella pyrenoidosa</i>	1.75	1.36	.39	22.3	11.5

Table 13.1 gives the experimental results together with the calculated values for Γ . In no case did the number of quanta used per CO_2 molecule reduced fall below nine.

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14.

The Photochemical Liberation of Oxygen From Water by Isolated Chloroplasts¹

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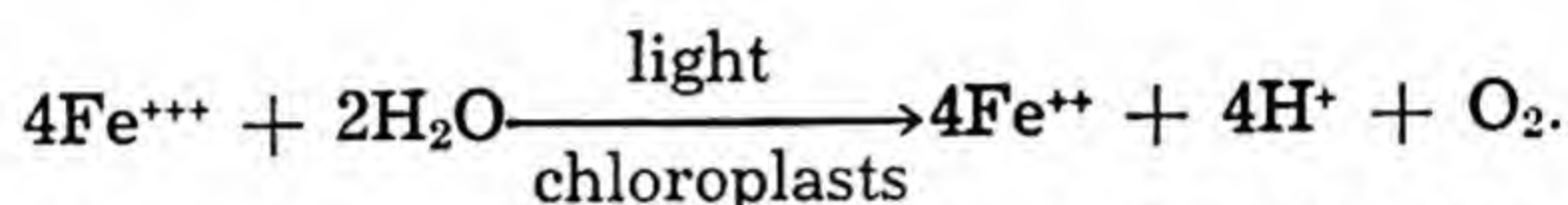
THE FACT that oxygen evolution occurs during the illumination of isolated chloroplasts, ground leaves, or even aqueous suspensions of powders obtained from dried leaves, has been known for approximately fifty years (1, 2, 3, 4) and has recently been confirmed by a new physical method (5). The amount of oxygen released in the experiments, however, was so small that extremely sensitive methods were required for its detection, and was very small compared with the amount evolved in normal photosynthesis. It is probable that the lack of progress in the study of photosynthesis compared with the advances made in the study of respiration is partially due to the fact that photosynthesis has not been found to occur apart from intact cells. In the study of respiration it has been possible to obtain cell-free, metabolically-active extracts from which enzymes could be isolated.

The first indication came in 1937 that cell-free extracts capable of effecting a portion of the over-all reaction of photosynthesis might be obtained and studied. At this time R. Hill reported that illumination of isolated chloroplasts, after the addition of aqueous extracts of acetone-treated yeast or leaves, resulted in oxygen evolution much greater than that ever previously found (6). The total amount of oxygen released was correlated, in the case of chloroplasts suspended in yeast extract, with the amount of iron salts of organic acids present.

By using certain complex ferric salts, such as ferric oxalate, Hill was able to dispense with the use of extracts from leaves or

¹This study was based on experiments done in the Department of Botany, University of Minnesota.

yeast. During this reaction ferric oxalate was reduced to ferrous oxalate, and oxygen was quantitatively determined as oxyhemoglobin. By the addition of ferricyanide as a secondary oxidant to oxidize the resulting ferrous oxalate, it became possible to measure oxygen production by the usual manometric methods (7). This is now known as the Hill reaction. It was believed at first that ferricyanide could not serve as an oxidant for the reaction in the absence of ferric oxalate, but this was later found not to be so (8, 9). The equation for the reaction with ferricyanide is as follows:



This work was spurred on by the belief that the reaction involves the mechanism of normal photosynthesis, which forms a reducing agent later used in CO_2 fixation and evolves O_2 as a by-product, and that it might serve as a test system and starting point for the isolation of some of the photosynthetic enzymes. It is important to bear in mind that no CO_2 fixation has been demonstrated by such illuminated suspensions of chloroplasts, even when subjected to examination with C^{14}O_2 (10). CO_2 fixation is presumed to be a process powered, in intact cells, by the normal reduction product of this first step.

That the evolution of O_2 by isolated chloroplasts is accomplished by the same mechanism as O_2 evolution in ordinary photosynthesis is illustrated by the following similarities:

(a) Oxygen is evolved and visible radiation is required, the region of the spectrum utilized being that absorbed by chlorophyll (11).

(b) No reaction occurs after a chloroplast suspension has been heated, indicating the presence of one or more thermolabile constituents (8, 9, 12).

(c) Light saturation has been attained, indicating the involvement of a Blackman reaction (12, 14).

(d) The quantum efficiency obtained with spinach or *Tradescantia* chloroplasts has been found in several experiments to be reasonably close to that obtained for photosynthesis (15).

(e) The splitting of water as the source of oxygen has been demonstrated for photosynthesis by several investigators who used O^{18} as the tracer (16, 17, 18). We have been able to demonstrate that the oxygen evolved by isolated chloroplasts also originates from water, which is in accord with the stoichiometry of the reaction (Table 14.1).

(f) Intact cells of *Chlorella pyrenoidosa* evolve oxygen quantitatively from ferric oxalate-ferricyanide solutions in the absence

of carbon dioxide, as shown in Figure 14.1. Similar results, recorded previously, have been found with *Chlorella* suspended in solutions of benzaldehyde and other reagents, and in solutions of *p*-benzoquinone (9, 19).

(g) The expected effects of certain reagents known to inhibit photosynthesis have been noted, although the results are not consistent among various investigators. Such reagents as phenylurethane, which interfere in photosynthesis with the transfer of

TABLE 14.1

THE ISOTOPIC COMPOSITION OF OXYGEN EVOLVED BY ILLUMINATED CHLOROPLASTS AS COMPARED WITH THAT OF THE SUSPENDING WATER

Water Used	Oxidant	Ratio $O^{18}O^{16}/O^{16}O^{16} \times 100$	
		Method of O_2 Production	
		Photochemical	Electrolytic
Normal.....	0.02 <i>M</i> ferricyanide	0.39	0.38
Normal.....	0.02 <i>M</i> ferricyanide	0.39	0.38
Enriched.....	0.02 <i>M</i> ferricyanide	1.4	1.3
Enriched.....	0.02 <i>M</i> ferricyanide	0.84	0.8
Enriched.....	0.0067 <i>M</i> quinone	0.62	0.61
Enriched.....	0.0035 <i>M</i> indophenol	0.57	0.62
Enriched.....	Dil. 2-6 dichlorophenol indophenol	0.54	(0.57-0.49)
Enriched.....	0.00221 <i>M</i> K_2CrO_4	0.52	0.49

energy from chlorophyll, affect the reaction of the chloroplasts similarly (9, 11, 20, 21). However, conflicting results with isolated chloroplasts have been obtained with the reagent hydroxylamine, which is believed to inhibit the oxygen liberating enzyme, and with azide. Both of these were stated by Hill (11) and by Aronoff (20) not to cause inhibition. The test reactions used by them were the ferric oxalate-hemoglobin method, and the manometric determination of oxygen with *p*-benzoquinone, respectively. Macdowall (21) found 50 per cent inhibition at 0.08 *M* azide or 3×10^{-4} *M* NH_2OH , measuring the initial rate of dye reduction. At the higher inhibiting concentration of this poison the effect on decreasing the final amount of dye reduced was much greater than the effects on the initial rate of its reduction.

Of the methods used to detect the inhibition it is probable that the photometric method with dye reduction is more reliable, since any changes in dye concentration resulting from interaction of the inhibitor is immediately detectable, except where the inhibitor itself can reoxidize the leuco dye. Using an air atmosphere in place of nitrogen and measuring O_2 production for periods up to an hour from the ferric oxalate-ferricyanide mixture, it was found (24) that

a 72 per cent inhibition with 10^{-4} M hydroxylamine occurred, and with 10^{-3} M azide the reaction stopped immediately and photooxidation with oxygen uptake took place. Cyanide inhibits a dark reaction of photosynthesis believed to be the carboxylation of CO_2 , and has no effect here as would be expected, since CO_2 is not involved (11, 21, 23).

The early work on this problem showed the necessity of conducting all the operations of isolating chloroplasts at a low tem-

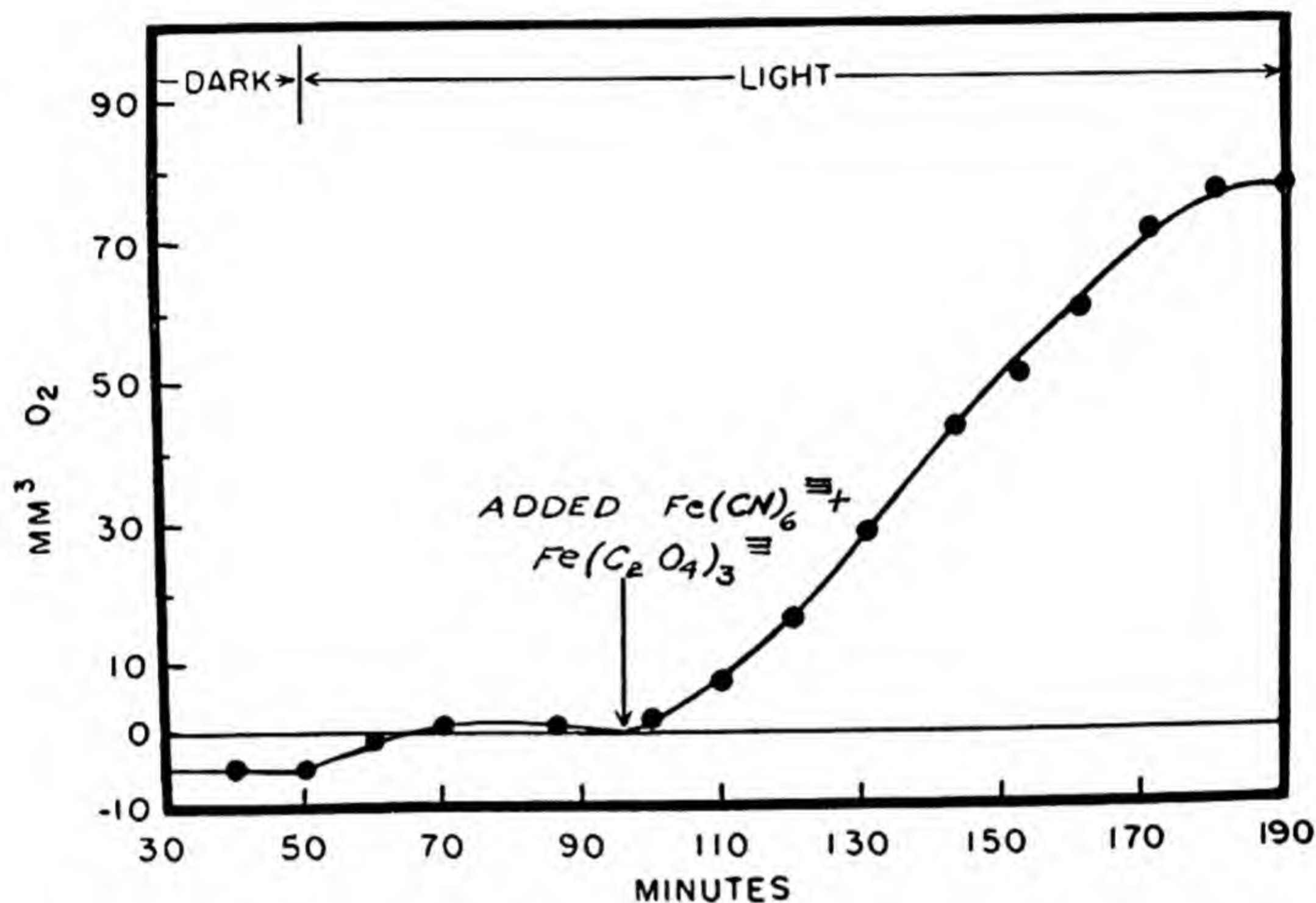


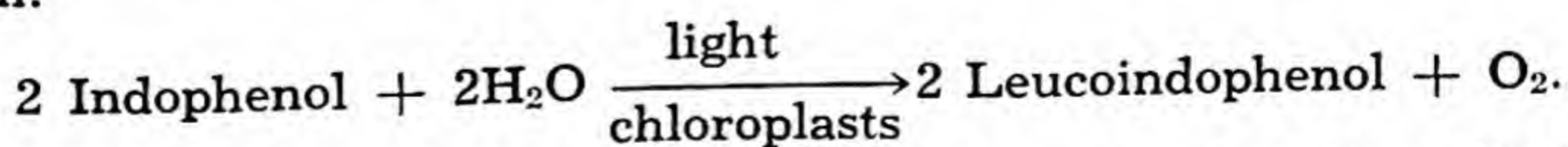
FIG. 14.1—Quantitative evolution of oxygen by *Chlorella pyrenoidosa* suspended in ferric oxalate-ferricyanide solution; 25°C ., CO_2 absent, N_2 atmosphere, $84 \text{ mm}^3 \text{ O}_2$ equivalent of ferricyanide present, yield = 93 per cent of theoretical.

perature (0°C .) to assure the obtaining of active suspensions. However, storage at 0°C . of such suspensions only reduced the rate of the activity loss during storage to about 50 per cent in 24 hours. Of a considerable number of substances tried as possible stabilizers during storage, 10 per cent propylene glycol was found to be the most effective (14). Good stabilization has also been obtained by Warburg and Lüttgens (9), using a mixture of 0.05 per cent KCl and 0.05 M K-phosphate (pH 6.5). Powders of chloroplast preparations obtained by lyophilization always show a much reduced activity, which continues to decline during storage (9, 22). Rapid drying effected by adding anhydrous sodium sulfate to the chloroplast suspensions at a temperature above the melting point of

$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ was followed by removal of the water of crystallization in vacuo at 0°C . This procedure yielded preparations still active, but no more so than those obtained by lyophilization (24), which usually results in a loss of about half of the activity and gives preparations that are difficult to resuspend. Chloroplasts stored both at 0°C . and at 35°C . showed an optimum stability at pH 6.5, and those kept at pH 5.1 or 7.4 lost half their activity in two hours at 0°C .

The optimum conditions found by Holt and French (8) for the manometric assay of activity in the ferric oxalate-ferricyanide mixture were 15°C ., pH 7.0 (0.063 M sodium sorbitol borate buffer), an inert gas atmosphere such as nitrogen, and 10 per cent NaOH in the inset of the manometric vessel to absorb any CO_2 that might result from a photodecomposition of oxalate. The use of temperatures higher than 15°C . caused a very rapid inactivation of the chloroplasts, and oxygen evolution ceased after a short time. With temperatures lower than 15°C . the rate remained constant for a longer time, and allowed a larger volume of oxygen to be evolved before the suspension was completely inactivated. A reduction in the length of time necessary to determine the activity of a preparation was accomplished by titrating, at constant pH, the hydrogen ion resulting from the reduction of the ferric salt (8). In this way it was shown that the temperature coefficients for oxygen evolution and for the inactivation of the chloroplasts during the reaction were 3.5 and 3.9, respectively, for the temperature range $3\text{--}15^\circ\text{C}$.

As a consequence of the finding that ferricyanide can replace ferric oxalate as the primary oxidant in the reaction medium, and of the report by Warburg and Lüttgens (13) that *p*-benzoquinone functions similarly, a survey of other oxidants was made. Oxygen evolution was observed manometrically when solutions of a few oxidation-reduction dyes or potassium chromate were used. Warburg and Lüttgens also report that β -naphthoquinone sulfonic acid can be used (9) as did Aronoff (20), who also found low rates with β -anthoquinone sulfonate. Bromate, chlorate, permanganate, nitrate, persulfate, dehydroascorbic acid, oxidized glutathione, coenzyme I, and cytochrome *c* were among those compounds tried which did not function as oxidants in this reaction. The volume of oxygen evolved when phenol indophenol is used corresponds to the equation:

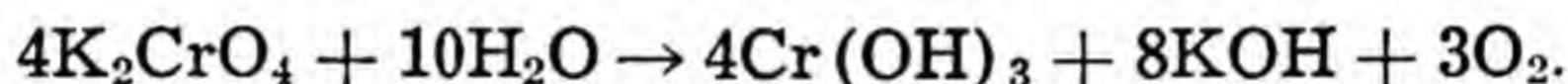


Since such oxidation-reduction dyes are reduced from a highly colored form to a leuco form, it is apparent that a very sensitive

test system for assaying the activity of dilute chloroplast preparations is available. The blue dye 2,6-dichlorophenol-indophenol could not be tested manometrically since it absorbs too much of the incident light, but dilute solutions are rapidly reduced. One can easily follow the disappearance of the oxidized form of this reagent visually from an initial concentration equivalent to 0.06 mm.³ O₂ per ml. to complete reduction in a mixture containing chloroplasts equivalent to a chlorophyll concentration of 0.0015 mg. per ml. (26).

For quantitative measurements of the dye reduction, a photometric method was devised. By the use of a recording apparatus it was possible to show: (a) the rate of reoxidation of the reduced phenol-indophenol is negligibly slow compared to its reduction by a chloroplast preparation of moderate activity; (b) the dye is not destroyed during the reaction since reoxidation by a small volume of H₂O₂ gave the initial reading obtained at the beginning of the illumination (14).

With chromate the yields of oxygen varied between 50–75 per cent of the theoretical amount, assuming the splitting of water and a reduction from Cr⁺⁶ to Cr⁺³ as in the following equation:



It was demonstrated that the chloroplasts suspended in chromate are not inactivated when the oxygen evolution ceases. The addition of ferricyanide-ferric oxalate mixture caused an immediate renewal of oxygen evolution as is shown in Figure 14.2.

Disintegrated chloroplasts obtained by such methods as treatment with supersonic vibration, prolonged maceration in a Waring blender, and by grinding have been shown to retain the ability of evolving oxygen (9, 11, 23). Hill (9) reported that preparations of disintegrated chloroplasts retained their activity for but two minutes, probably because low temperatures were not used in his procedures.

The retention of activity by the chloroplast material after disintegration is most important, since eventually a reduction to molecular dimensions is essential if one is to separate the individual components involved. Following high speed centrifugation, such a preparation made by supersonic treatment shows a Tyndall effect, but shows no apparent visible structure when examined microscopically. By using fragmented chloroplasts, Warburg and Lüttgens (25) showed that repeated washing and centrifugation with distilled water caused a loss of activity which can be restored completely by the addition of Cl⁻ or Br⁻, and less effectively so by NO₃⁻ or I⁻. They consider Cl⁻ to be a coenzyme of photosynthesis. This effect of Cl⁻ has been shown also with washed preparations for

the conditions where ferricyanide or the indophenol dyes are used for activity determinations (14).

Various treatments of fragmented preparations such as salt precipitations, pH precipitation, adsorption on various types of columns, and differential centrifugation have not yet yielded encour-

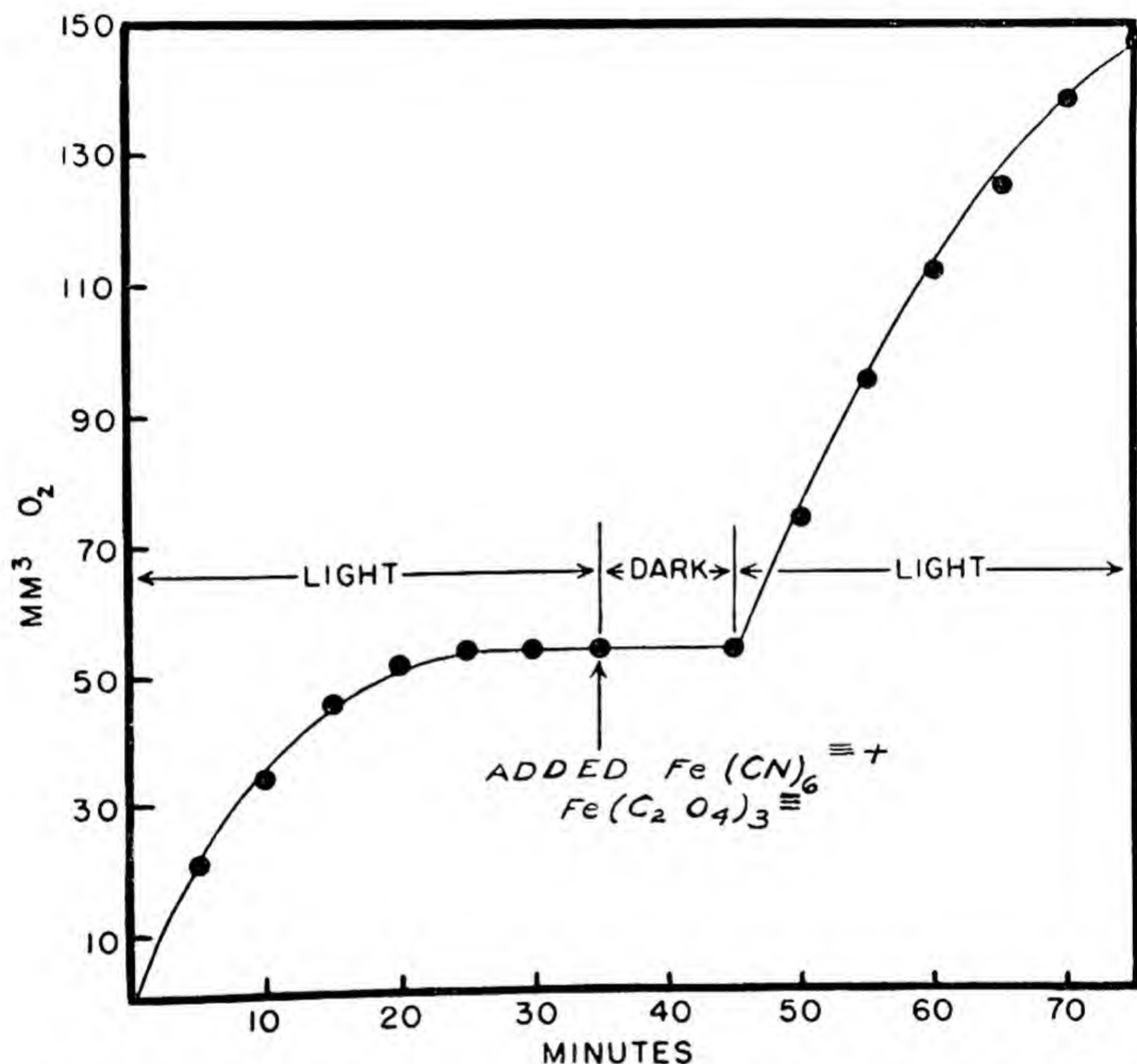


FIG. 14.2—Evolution of oxygen by isolated chloroplasts suspended in potassium chromate solution.

aging results in the isolation of individual enzymes or fractions which show considerable enhancements of activity (9, 14).

A suspension of chloroplasts in 10 per cent propylene glycol was treated with supersonic vibration and then separated into fractions of various sized particles by centrifugation (14). The first precipitate, which contained half the total nitrogen present, was obtained after 5 minutes at 7,600 x g. Its activity was lower than that of the starting material, while the supernatant had twice

the original activity per mg. of N. This supernatant was recentrifuged 10 minutes at 8,600 x g., again sedimenting about half of the nitrogenous material. The activity was equally divided between the precipitate and the supernatant, and was about equal to that of the first supernatant. A final separation at 9,200 x g. for 30 minutes again divided the nitrogenous material about equally, and the activity of the sediment was 3.4 times that of the original material, while the supernatant was about half as active as the original preparation. Thus it appears that the more highly active constituents are in the particles sedimented in 30 minutes at 9,200 x g. but not in 10 minutes at 8,600 x g.

Warburg and Lüttgens discovered that the quantity of o-phenanthroline required to cause 100 per cent inhibition is that which would be just needed to combine completely with the amount of zinc (0.0068%) found by elementary analysis of chloroplast fragments (9, 13). The inhibition is reversed by adding zinc sulfate.

That the evolution of oxygen does not proceed through the decomposition of H_2O_2 by catalase is evident from its lack of sensitivity to cyanide. Furthermore, this is shown also by the fact that peroxide is not indicated in the equation for the reaction, and by the finding (24) that the catalase activity of chloroplasts can be completely inhibited by concentrations of azide which have little effect on the chloroplast reaction.

Macdowall (21) found stimulation at low concentrations of ether, thymol, chloroform, and especially strychnine. For the concentrations 5×10^{-5} M to 5×10^{-3} M stimulations of 23—94 per cent were noted with strychnine. He attributed these results to the possible removal of a natural inhibitor. The increased rate of dye reduction was demonstrated not to be the result of a reduction or decomposition of the dye by strychnine.

It would seem that the immediate problems which should be solved before the active components can be isolated are stabilization of chloroplast preparations for long periods of storage, and methods for more complete disintegration of chloroplasts, leaving the activity unchanged. The isolation of the active substances should lead to further clarification of the role of chloride as a coenzyme and of zinc as a heavy metal catalyst.

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15.

Chlorophyll Fluorescence as an Energy Flow Meter for Photosynthesis

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DURING the years the author was a co-worker of the biophysical research group at Utrecht, Netherlands, many combined observations of photosynthesis and fluorescence of various monocellular organisms under a wide range of circumstances were made (1). The following picture was found to be in good agreement with the experiments and is considered a working hypothesis for the photosynthetic process.

A. Purple Sulfur Bacteria and Similar Organisms

1. A hydrogen donor, supplied to the cells externally, is converted by some enzymatic dark process into an energy acceptor. This energy acceptor contacts the chlorophyll.

2. When chlorophyll absorbs quanta of light, the excitation energy, apart from losses as heat or fluorescence, is under normal photosynthetic conditions transferred to this energy acceptor.

3. The energized acceptor then reacts at another dark catalytic surface with CO_2 (or a product derived from CO_2) reducing the latter to carbohydrate.

B. Chlorella and Other Green Plant Cells

The same scheme is followed, except that the hydrogen donor is water and is always present in abundance.

It is assumed, in agreement with current physical ideas, that the rate of fluorescence is a constant (very small) fraction of the concentration of excited chlorophyll, because fluorescence is a spontaneous process governed by a constant transition probability. The concentration of excited chlorophyll is determined by the rate of excitation due to absorption of light quanta and the rate of destruction of the excited state (for which we shall introduce the

term "deciation") due to; (a) return of the excited electron to its ground state with formation of heat, (b) fluorescence, (c) transfer of energy to the photosynthetic energy acceptor, and (d) transfer of energy to other energy acceptors (if present).

For a given rate of excitation (light intensity) the rate of deciation determines the equilibrium concentration of chlorophyll, hence the fluorescence intensity. A higher rate of fluorescence corresponds to a smaller rate of deciation and vice versa. Thus, we conclude that the fluorescence is a measure of the transfer of energy to the photosynthetic energy acceptor, provided we can either neglect the other ways of deciation or keep them constant or controlled.

We shall now briefly review the principal reactions of fluorescence and photosynthesis to various agents, and the interpretation of these effects in terms of the above scheme.

In the first place, a number of agents influence the supply of energy acceptor to the chlorophyll, particularly in the case *A* of purple sulfur bacteria. For high concentrations of hydrogen donor, the capacity of the dark reaction that transforms the donor into an energy acceptor appears to limit the rate of supply. This limitation is dependent on temperature, on the kind of hydrogen donor used, and on pH. If the donor concentration is very low, it appears that its concentration is the limiting factor for the rate of supply of the energy acceptor to the chlorophyll. We feel justified in making such inferences without knowing any more specific details about the dark reaction, the chemical constitution of the energy acceptor, etc. merely from a thorough study of the observations of photosynthesis and fluorescence in relation to all relevant variables of the problem. A few examples of how such conclusions are drawn will be briefly outlined.

It is adequate for this purpose to consider the differential photosynthetic or fluorescent efficiency (i.e., the slope of a plot of the rate of photosynthesis or fluorescence versus incident light intensity) as a function of the incident light intensity for various concentrations of hydrogen donor. The photosynthetic differential efficiency (*pde*) is practically independent of the hydrogen donor concentration at low light intensities. Of course, at very high light intensities it is also constant, namely zero, on account of the saturation of photosynthesis. However, the transition from the low intensity value to zero occurs at a light intensity which increases with increasing donor concentration up to a certain maximum value. It cannot be made to exceed this value and becomes independent of further increase in the external concentration of hydrogen donor.

The fluorescence differential efficiency (*fde*) is independent of the hydrogen donor concentration at low light intensities, and also

at very high ones, where the *fde* is governed by the loss reactions only. Its value at high light intensities is about four to five times as high as at low ones. However, the transition from the low light intensity value to the high intensity one occurs at a light intensity which is practically identical with that where the transition of the *pde* occurs.

These observations would still leave room for a multitude of different explanatory schemes, but the majority of these are ruled out by the additional observation that absence of CO_2 , which, of course, stops photosynthesis, leaves the *fde* practically unaffected. It will readily be seen, however, that the scheme outlined in A calls for just such dependences. If CO_2 is abundant, and if its dark reactions do not limit the rate of photosynthesis, one may expect either limitation by the light intensity if the acceptor supply is plentiful, or limitation by the supply of energy acceptor if the light intensity is high. The fact that an unlimited increase of external hydrogen donor concentration does not increase the latter limits in proportion, proves that the hydrogen donor, as such, is not identical with the energy acceptor but has to be derived from it by a dark reaction of limited capacity. The fact that abundance or absence of CO_2 leaves the fluorescence practically unaffected proves that the reactions of the energized acceptor subsequently take place in another catalytic system which hardly reflects its reaction rate on the transfer system chlorophyll-acceptor.

In a similar way, by confrontation of various sets of observations, the other conclusions have been drawn. For a detailed discussion the reader is referred to the Utrecht communications (1). In photosynthesis of green plant cells using water as their hydrogen donor most of the features discussed are not observable. This is true partly because the hydrogen donor seems to be present always in unlimited supply; partly, also, because it seems that in these cases the maximum rate of the supply reaction of energy acceptor is higher than the maximum rate of operation of the CO_2 system, so that the former does not constitute a limitation for photosynthesis under most circumstances.

In the second place, other substances than those prepared from the hydrogen donors for the photosynthetic process may also accept some of the energy from the excited chlorophyll. Narcotics like ethyl urethane appear to act in this way. They decrease the *pde* in the light limiting range and change the *fde*, tending to make it more uniform at all intensities, especially when the narcotic concentration is high.

In the third place, a number of agents act on the system where the energized acceptor reacts with CO_2 or its derivatives. Particularly temperature, low CO_2 supply, and KCN inhibition belong to

this class. For a discussion of these variables the reader is referred to the literature (1).

This paper aims to show that by working out certain details concerning the excited chlorophyll, but without altering essential features of the scheme set forth, a still larger domain of observations may be covered. These observations are related to the "photosynthetic unit" (2). To this end we shall try to trace the history of the energy between its absorption in chlorophyll and its transfer from chlorophyll to other systems.

Since the chlorophyll molecules are bound physically or chemically to much larger protein complexes whose structure is probably periodic, it is likely that the chlorophyll is also arranged in a periodic pattern or structure. It is by no means necessary to assume that such a structure is similar to that of pure solid chlorophyll. On the contrary, it is in agreement with current ideas that the properties of such an adsorbed structure, e.g., symmetry and periodicity, are determined to a great extent by the protein carrier. In this restricted sense then the chlorophyll may be considered as a two-dimensional crystal.

If one assumes that average grana in *Chlorella* (3) have a diameter of 0.3μ and that the lattice constant of the two-dimensional chlorophyll lattice is of the order of 25 \AA , which would be in agreement with Hubert's ideas, then the number of chlorophyll molecules per monomolecular layer is

is $\frac{\pi}{4}(120)^2 \sim 11,000$. This is of the

same order of magnitude as the photosynthetic units postulated by Wohl (2) and investigated by Emerson, *et al.* (4). Thus we may say that a photosynthetic unit may well be made up of all chlorophyll molecules of one monomolecular chlorophyll layer through a granum.

The physical properties of such periodic structures have received much attention in recent years. Although most of the theoretical work refers to three-dimensional lattices, the essential properties are easily verifiable for the two-dimensional case (5,6). Theoretically it appears that two types of processes may, in principle, occur in the chlorophyll when light quanta are absorbed. It seems impossible at present to decide from known experimental data which one is actually operating; therefore, a discussion of both will follow.

The first possibility is the formation of an exciton. A classical description of an exciton would state approximately that an excited chlorophyll molecule may transfer its excitation to its neighbor, being left in the ground state itself. If this transfer occurs relatively easily, the state of excitation may diffuse through the crystal by

repeated transfer, although no particles diffuse through the crystal with it. Quantum mechanically the exciton is not necessarily localized at any instant at a given chlorophyll molecule but is spread out over the whole crystal. This picture is very similar to the general one given previously. It merely contains a justification from crystal physics for the postulated processes of a photosynthetic unit, as well as the various types of deactivation mentioned at the outset, but no estimate of the relative probabilities of these types is as yet possible.

The second possibility is to assume that the two-dimensional chlorophyll "crystals" are photoconductive. The absorption of a light quantum lifts one electron into the conduction band and leaves one hole in the ground band. Electron and hole are free to move around, although their mobilities may differ; or they may be considered to be spread over the whole crystal, from a quantum mechanical viewpoint. Further assumptions concerning the fate of the conduction electrons and holes must check with a number of experimental facts which permit defining the scheme more accurately.

An important fact is the observed proportionality between fluorescence and exciting intensity in the range of light limitation. This requirement can be satisfied along conventional lines only if one makes the reasonable assumption that the rates of loss of electrons and holes with formation of heat or fluorescence are proportional to the product of their concentrations, and the implausible assumption that electrons and holes are transferred to other systems at rates proportional to the *square* of their concentrations. This would mean effectively that transfer of electrons to photosynthetic acceptors can proceed only with pairs of electrons, and similarly, that the holes can be operative only in pairs. Such an assumption is implausible qualitatively because it may be expected to yield a rather low efficiency for the photosynthetic process. Whatever the exact value of the quantum efficiency may be, it is known to be a rather efficient process, hence the scheme cannot well be maintained in this form. However, a more consistent modification of the electron and hole scheme is possible and will now be described.

Assume that the time between successive absorptions of exciting light quanta in the same crystal unit (interquantum time) is, for all intensities concerned, greater than the lifetime of an electron in the conduction band and greater than that of a hole. The effect of one absorbed quantum is then disconnected from that of the next quantum. The probability per unit time for recombination of electron and hole with formation of heat or emission of a fluorescence quantum is proportional to the intensity of the exciting light and to the lifetime of the shortest living of the two. In other words,

this scheme can be made to agree with observations such as those communicated for purple sulfur bacteria by assuming: (a) the electrons can be transferred to an electron acceptor which is thereby effectively reduced and can now react with CO_2 or its derivatives to yield carbohydrate, (b) the holes can be transferred to a hole acceptor (a molecule that can transfer one of its own electrons into the hole of the unit) which is thereby effectively oxidized and can now react with the hydrogen donor or its derivatives, or is possibly identical with these, (c) the lifetime of the holes is shorter than that of the electrons, under normal photosynthetic conditions.

One can easily verify that, in this scheme, fluorescence reacts to a low supply of hydrogen donor in agreement with the observations stated for purple sulfur bacteria. It is also evident that a low supply of CO_2 will increase the lifetime of the electrons without affecting fluorescence, since the lifetime of the holes remains shorter. The other relations between photosynthetic rate, fluorescence, and other variables fit into this scheme with equal simplicity. For *Chlorella* it is possible to adapt the scheme by assuming that limitations of the rate of donor supply are irrelevant because other factors limit sooner, just as was assumed previously.

The obvious advantage of this scheme is that it pictures how the light gives rise to a reducing agent (electron) and an oxydizing agent (hole) both of which are mobile and will move in their own way, performing oxidation and reduction at different places. The exciton or energy transfer scheme on the other hand might energize almost any type of reaction and, therefore, necessitates rather detailed assumptions about the chemical nature of the energy acceptor. Since, however, these assumptions have, so far, not contradicted any experiment, no decision between the schemes can be made until further information is available.

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16.

The Relation of the Fluorescence of Chlorophyll to Photosynthesis

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OBSERVATIONS of variations in the intensity of the fluorescence of chlorophyll in photosynthesizing plants and bacteria enable us to draw conclusions concerning the manner in which the light energy absorbed by the chlorophyll is used for the reduction of CO_2 . From these observations answers to such questions as the following are available:

Does the process of reduction of CO_2 take place through direct contact of CO_2 , or of some complex molecule in which CO_2 has been fixed, with the light-excited chlorophyll itself, or does the process consist of a photochemical production of a reducing agent which then reduces CO_2 by non-photochemical enzymatic reactions?

Are the photochemical steps in photosynthesis in green plants different from the photochemical steps of the process of photo-reduction in green algae and purple bacteria?

How are the transient rises of intensity in fluorescence of chlorophyll connected with the variation of the photosynthetic rate during the induction period?

How does the plant protect itself against damaging photochemical reactions, such as photooxidation, and against the overproduction of photosynthetically formed substances?

Answers based on measurements of variations in fluorescence intensities have, of course, the disadvantage that they depend upon indirect evidence. It is, therefore, necessary to compare fluorescence intensity with photosynthetic rates in whole plants, in chloroplasts, and in bacteria under a great variety of conditions, and to make the greatest possible use of all information concerning relations between the fluorescence intensity and photochemical activity of complex dyes *in vitro*.

Kautsky and co-workers first pointed out that typical variations in the fluorescence intensity accompany the variations in the photosynthetic rate which are observed during the induction period. Since that time careful measurements of the fluorescence intensity of chlorophyll and comparisons with corresponding rates of photosynthesis have been made by many observers. However, only two detailed theoretical interpretations of the data have been worked out. The one, representing the views of a group of Dutch observers, Katz, Wassink, Dorrestein, etc., is briefly surveyed by Katz in the preceding paper, "Chlorophyll Fluorescence as an Energy Flow Meter for Photosynthesis." The other has been developed by the writer, together with a group of co-workers. It is evident that a satisfactory interpretation must be in agreement with all available evidence and able to provide answers to a maximum number of problems with a minimum number of assumptions. In the present paper the theories of the Dutch group and of the Chicago group will be compared, and reasons will be given to show why the author prefers the latter point of view.

RELATION BETWEEN FLUORESCENCE OF DYES AND SENSITIZED PHOTOCHEMICAL REACTIONS *IN VITRO*

Dyes which fluoresce in solution are generally sensitizers of photochemical reactions, but the relation between these two properties is not so simple that dyes having the highest fluorescence yield are necessarily the best sensitizers. Because they are poly-atomic, all organic dyestuffs such as chlorophyll can, after the absorption of light, dispose of the excitation energy of their electronic system in several ways. The energy may be re-emitted as fluorescence or it may be used for radiationless transitions of the electronic system. If the exciting light belongs to the visible part of the spectrum, only two types of radiationless transitions occur in dye molecules dispersed in condensed systems. They are: (a) transitions of the electronic system into lower quantum states by the process of internal conversion, whereby electronic energy is converted into oscillation energy (heat) of the atomic constituents of the dye molecule or of the complex to which the dye is attached; or (b) transitions into a changed pattern of the electronic bonds of the atoms of the dye or of other molecules of the complex, resulting in chemical reaction.

It is obvious that the smaller the probability of radiationless transitions during the lifetime of the excited state the higher will be the fluorescence yield. Since the maximum fluorescence yield of chlorophyll is of the order of magnitude of a few per cent in

organic solvents, and of one-tenth of one per cent in plants and purple bacteria, practically all of the light energy absorbed must be used in radiationless transitions. Certain photooxidation reactions take place in organic solvents with a quantum yield of ~ 1 , and there are good indications that the yield is also ~ 1 for each photochemical partial reaction of photosynthesis in plants. These quantum yields indicate that if substances able to use the light energy for chemical purposes are present in suitable concentrations, the energy is used practically *in toto* for chemical change.

The simplest interpretation of these facts is the assumption that in the presence of molecules able to use the energy for chemical reactions, the energy transfer from the excited dye to these molecules will occur before internal conversion takes place in the dye molecule itself. This leads to the conclusion that the fluorescence should be quenched by the addition of acceptor molecules, just as is the case for the fluorescence of diatomic molecules. Actually this conclusion is not in accordance with observations of the fluorescence of chlorophyll *in vitro*. Furthermore, a more detailed analysis shows that it would not be expected to be generally true. Briefly, the reasons are as follows: Radiationless transitions in polyatomic molecules can occur only when a certain critical constellation of atomic positions and energy distribution has been reached through movements of the atomic constituents and fluctuations of atomic oscillation energy in the molecules. The time needed to reach this critical point depends not only upon the structure of the excited dye molecules but also upon the structure of the molecules coupled to them. If the acceptor molecules are themselves polyatomic, the formation of short-lived or permanent complexes between dye and acceptor molecules makes the structure of the dye effectively more complex. In general, this prolongs the time needed to reach the critical point.

However, even if in the complex of dye and acceptor the chemical transition precedes the internal conversion, it is possible that it will take longer to reach the critical point for the chemical transition in the complex than it will to reach the critical point for internal conversion in the isolated dye molecule. In such a case the fluorescence yield would be increased by addition of polyatomic acceptor molecules. On the other hand, if the dye is already connected with a very complex molecule such as protein, the addition of a smaller complex molecule may have little influence, and it cannot be predicted whether the fluorescence intensity will rise a little or fall a little. These considerations are not only theoretical; they are supported by many observations showing that the coupling of dye mole-

cules to highly complex systems, such as solid surfaces, proteins, or solvents of high viscosity, considerably delays internal conversion and thereby strongly enhances fluorescence yields.

There are also observations which indicate that use of excitation energy for chemical change with quantum yields of ~ 1 does not necessarily have to precede internal conversion; rather, it may in some case be a consequence of it. Photooxidation reactions taking place in organic solvents in the presence of chlorophyll may be an example of this type. The fluorescence yield in such reactions is independent of the concentration of the substance being oxidized and of the oxygen concentration up to the concentration needed for photooxidation with a quantum yield of ~ 1 . A surplus of oxygen, however, quenches the fluorescence somewhat. A detailed analysis shows that two probable ways exist by which the excitation energy may be used for chemical purposes after internal conversion has been accomplished. The internal conversion of the electronic energy may result in the formation of either a lower but still energy-rich metastable state of the electronic system or of a short-lived energy-rich tautomeric form of the dye. A much less likely possibility involves reaction between the solvent molecules and the excited dye molecule.

These discussions may suffice to show that one cannot assume *a priori* that a lack of acceptor molecules for the energy absorbed by the chlorophyll in photosynthesizing cells must be a sufficient reason for a strong rise in the fluorescence intensity of the chlorophyll. Such an assumption is the fundament upon which Katz and co-workers base all their conclusions concerning the relationship between photosynthesis and fluorescence intensity.¹ On the other hand, the relation between the fluorescence of chlorophyll to photochemistry *in vitro* may be different from that in plants. Any assumption has, therefore, to be tested carefully by experiments with photosynthesizing cells before it can be accepted or rejected. In the following section experimental observations with plants and purple bacteria will be discussed which, according to the opinion of the writer, speak against the hypothesis of Katz and his group.

¹ Livingston and co-workers show, in a paper to appear soon in the Journal of the American Chemical Society, that the fluorescence yield of chlorophyll dissolved in certain hydrocarbons is increased a hundred or more times by traces of water in the solvent. Oxygen was absent and no photochemical reaction was found in either the dry or the wet solution. These observations offer new and convincing evidence for the statement that the fluorescence yield of polyatomic dyes is far more influenced by competition between internal conversion and light emission than by competitive utilization for photochemical reactions.

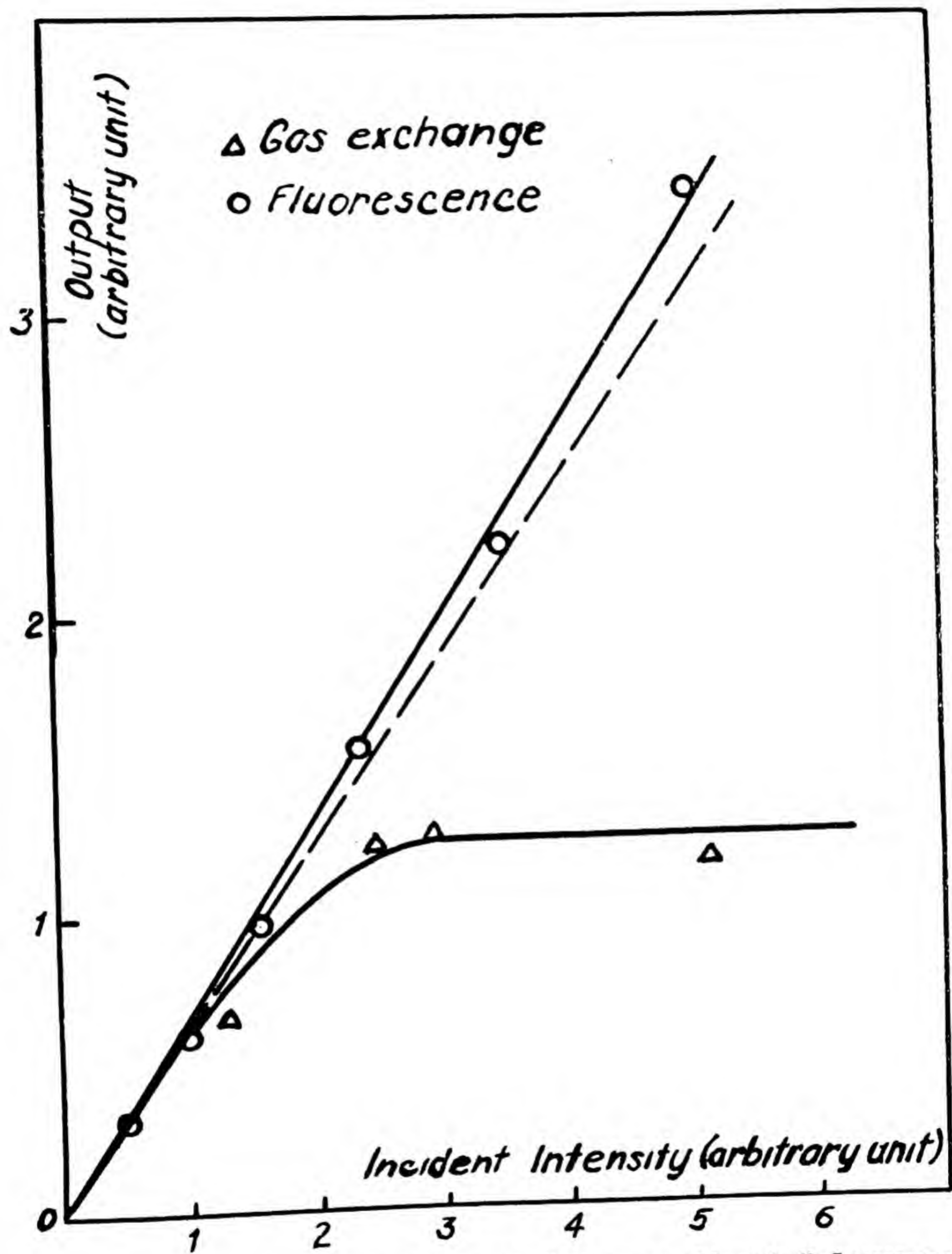


FIG. 16.1—Average curve of CO_2 assimilation and chlorophyll fluorescence in relation to incident intensity in suspensions of *Chlorella*.

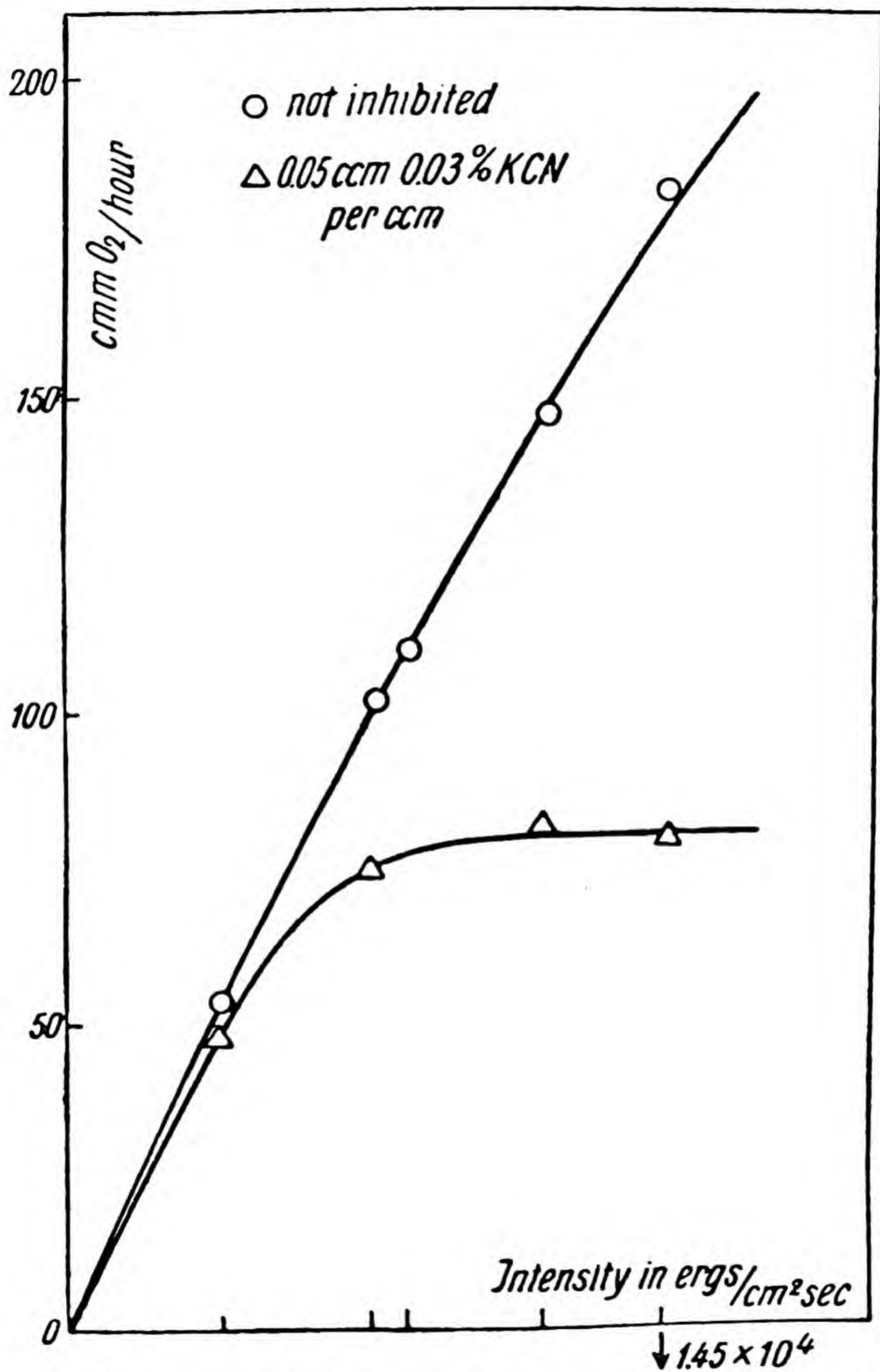


FIG. 16.2—Influence of cyanide upon the CO₂ assimilation in suspensions of *Chlorella*.

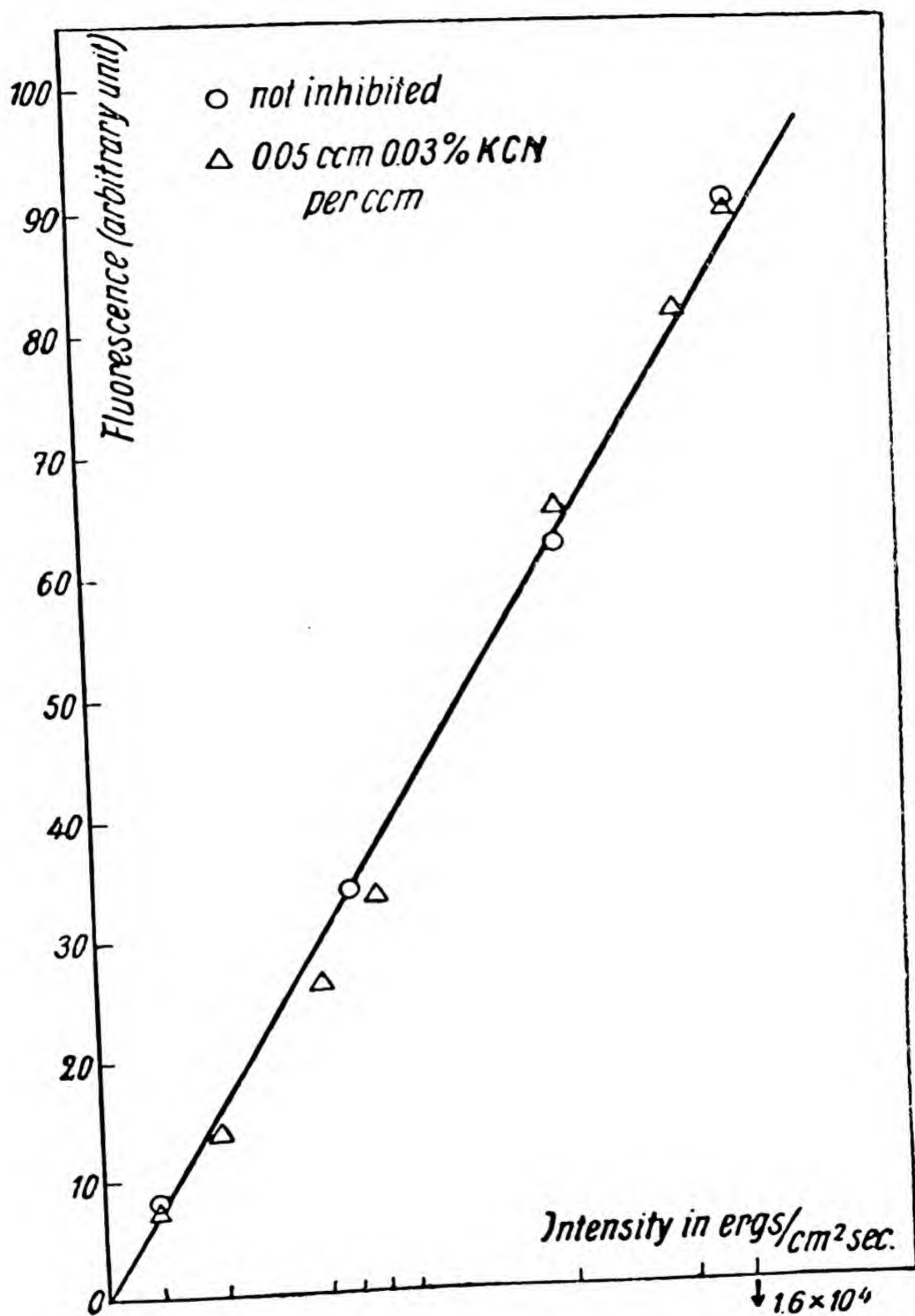


FIG. 16.3—Influence of cyanide upon fluorescence of chlorophyll in suspensions of *Chlorella*.

THE STEADY STATE OF FLUORESCENCE IN GREEN PLANTS

Ornstein, Wassink, and Katz rejected all kinetic theories of photosynthesis containing the assumption that CO_2 —or CO_2 attached to a more complicated molecule—is reduced in contact with chlorophyll. They took this stand because they did not find an increase in the fluorescence yield in the region of light saturation. They expected that a strong increase should occur at saturation if, from lack of CO_2 molecules or complexes, the excitation energy of the chlorophyll was not converted into chemical energy. They, therefore, introduced the hypothesis that a substance present in abundance is reduced at the chlorophyll, and that this substance then reduces CO_2 by an enzymatic reaction occurring apart from the chlorophyll. This hypothesis, however, needs to be supplemented by the assumption that the primarily reduced substance has a short lifetime and can vanish by back reactions. Otherwise the uptake of CO_2 by the plant would continue for a long time in the dark after the plant was exposed to high light intensities.

The discussion in the introduction has shown that we may expect small changes of the fluorescence yield, if any, when the surface of such a complex system as the chlorophyll-protein complex is denuded of substances which can use the excitation energy for photochemical processes. Furthermore, according to Franck and Herzfeld's theory (5), in the presence of enough CO_2 and at temperatures between 20° and 35°C ., the enzymatic formation of CO_2 complexes is so rapid that it can contribute very little to the limitations responsible for saturation. The lack of CO_2 complexes can become the main limiting factor only if their rate of formation is decreased by reduction of the CO_2 concentration, by poisoning the enzyme involved by cyanide, or by using low temperatures.

To test whether denudation of the chlorophyll by removal of CO_2 complexes causes changes in the fluorescence yield, it is necessary to find out whether the relation between the intensities of the fluorescence and of the exciting light is strictly linear or whether deviations from linearity, even small ones, occur in the region of intensities sufficient for saturation of photosynthesis. Figures 16.1, 16.2, 16.3, and 16.4 show observations made by Katz, Wassink, and co-workers (1, 2) with the alga, *Chlorella*. From these they deduce not only that the fluorescence intensity rises linearly with the intensity of the exciting light under normal conditions, but that the linearity is unaffected by the presence of moderate amounts of cyanide or by temperatures as low as 10°C . In Figure 16.1 the writer has added a dotted line drawn through the first three points of the fluorescence curve to show that rela-

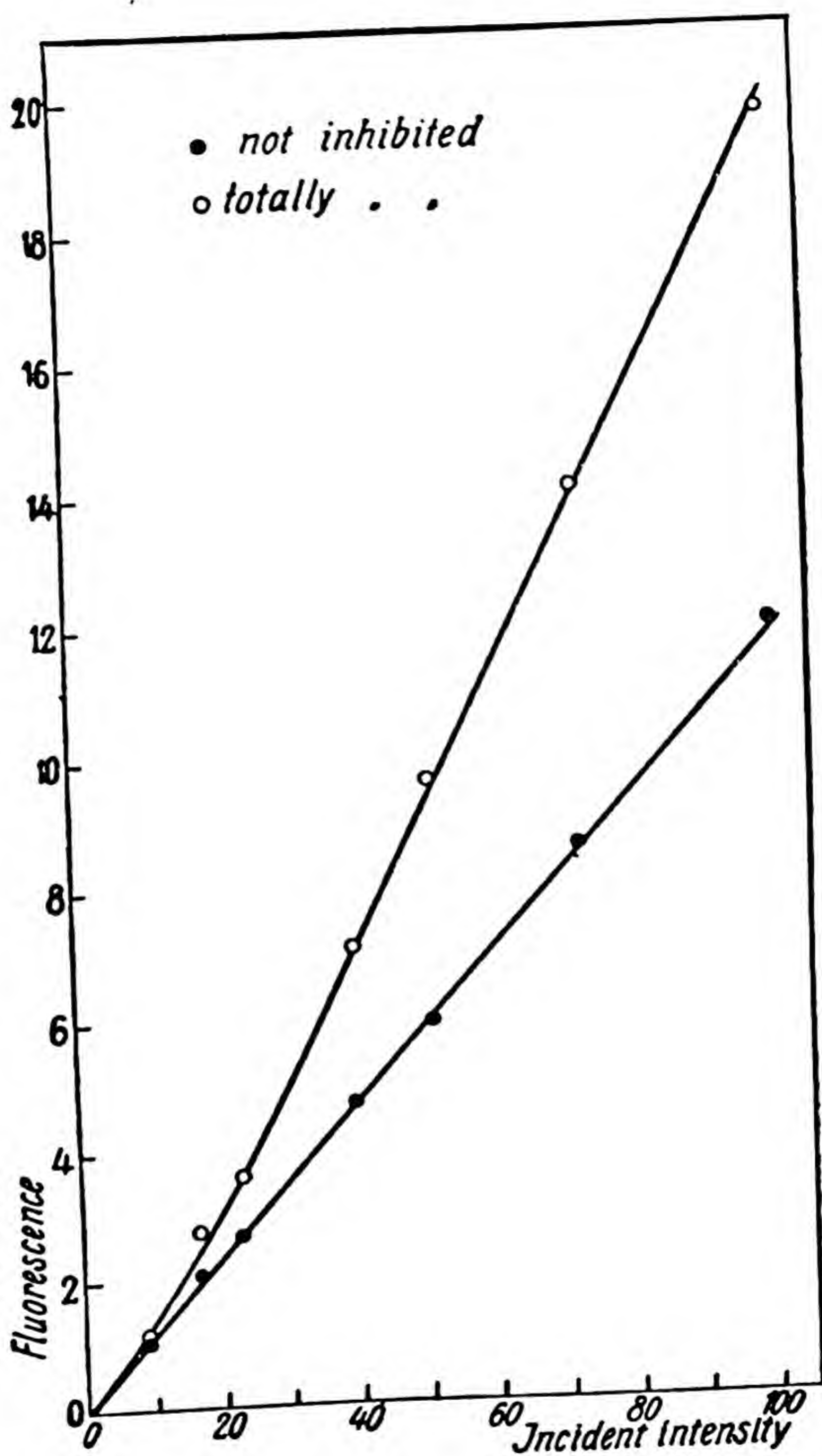


FIG. 16.4—Stationary values of chlorophyll fluorescence as a function of light intensity, with and without inhibition of photosynthesis by cyanide (gas phase air, temperature = 29°C.).

tively small deviations of the curve from linearity are by no means excluded by these observations. The observations of Shiau and Franck (8) of fluorescence intensity in the algae, *Chlorella* and *Scenedesmus*, measured under a variety of external conditions (see Figs. 16.5 and 16.6a) all clearly show deviations from linearity. The start of the curvature is shifted toward lower intensities by all influences which cause similar shifts of the saturation intensity.

According to our opinion, a comparison of the results of the Dutch group with those of other observers indicates unmistakably that such deviations do occur, and that the accuracy which Katz, Wassink, and co-workers regarded as sufficient is not great enough to detect them. Small differences in the shape of the fluorescence curves obtained from *Chlorella* raised in Holland and that raised in Chicago are quite possible, since the relative concentrations of the various enzymes involved in photosynthesis are known to vary with culture conditions, age of the plant material, etc. However, the fact that the Chicago algae show a nonlinear fluorescence curve under all conditions while the Dutch algae are supposed to give linear curves cannot be explained by such differences.

Further proof that curves of fluorescence yield versus exciting light intensity are not linear is available in the studies of McAlister and Myers (4) with leaves of young wheat and of Franck, French, and Puck (6) with leaves of *Hydrangea*. The use of leaves of higher plants is an advantage, in this particular case, because the CO_2 concentration necessary to reach the highest saturation rates and the saturation intensity lies much higher than in algae.

Experiments with *Hydrangea* in air with its natural CO_2 concentration have shown that, in the region of low light intensities where the rate of photosynthesis is proportional to the intensity of the incident light, the curve of fluorescence yield versus exciting light intensity rises linearly. At medium light intensities, where the photosynthetic rate curve starts to bend over toward saturation, it departs from linearity because the fluorescence yield starts to increase with the exciting light intensity. Finally, in the region of full saturation, it becomes linear again, but with a considerably higher slope than at low intensities.

Figure 16.7 presents an example of such observations. Since one curve is measured at 23°C . and the other at a temperature slightly above 0°C ., Figure 16.7 may also serve as an example of the shift in the point of departure from linearity caused by a lowering of temperature. The curve measured by McAlister and Myers, using young wheat plants, shows (Fig. 16.8) very clearly the dependence upon the CO_2 concentration of the region where the curve of fluorescence intensity versus exciting light intensity starts to depart

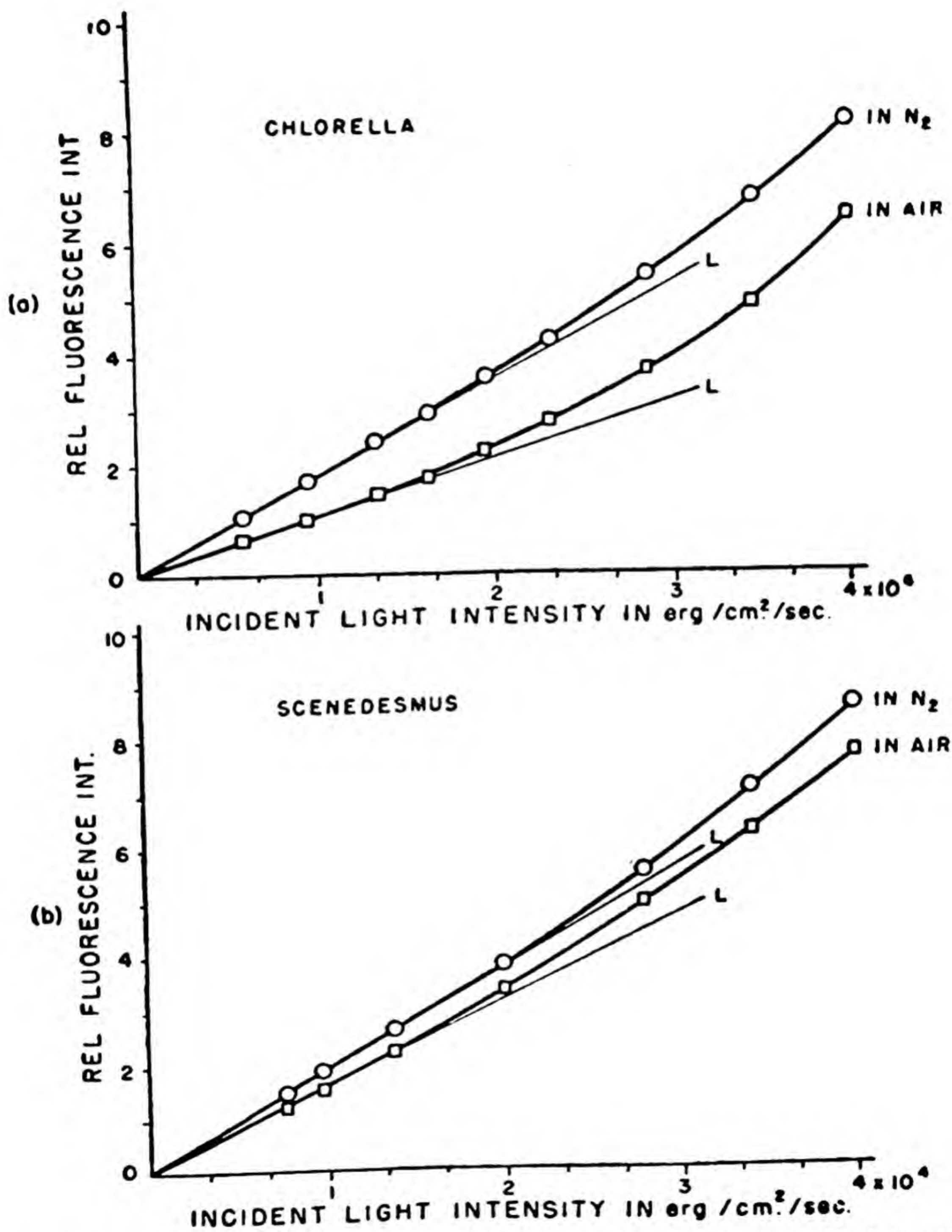


FIG. 16.5—Steady state fluorescence intensity vs. incident light intensity of (a) Chlorella and (b) Scenedesmus at 25° C., showing the increase in the fluorescence yield at high light intensities.

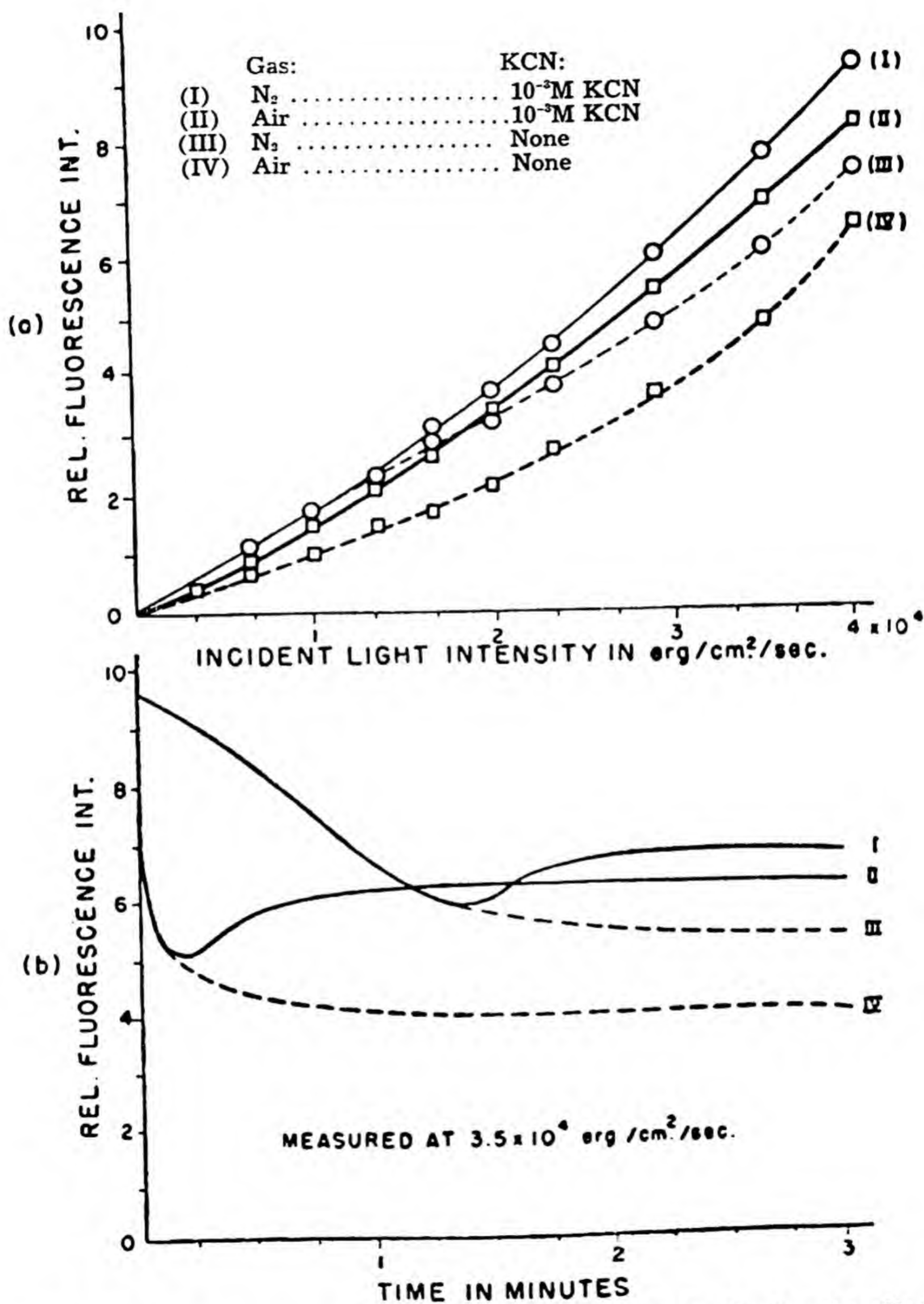


FIG. 16.6—Fluorescence behavior of *Chlorella* at 24° C., showing the effect of KCN in air and in nitrogen.

from linearity. With 4 per cent CO_2 the curve remains linear over the whole intensity range (saturation was not attainable in that case with the maximum light intensity available—about 80×10^4 erg/sec. cm^2), while with 0.03 per cent of CO_2 , the concentration normally present in air, the deviation starts at a light intensity of about 15×10^4 erg/sec. cm^2 .

Figure 16.9, taken from the same paper, shows that with another wheat plant the fluorescence curve in air actually starts to bend upward just where the photosynthetic rate curve starts to bend in the opposite direction. Note, however, that in nitrogen containing

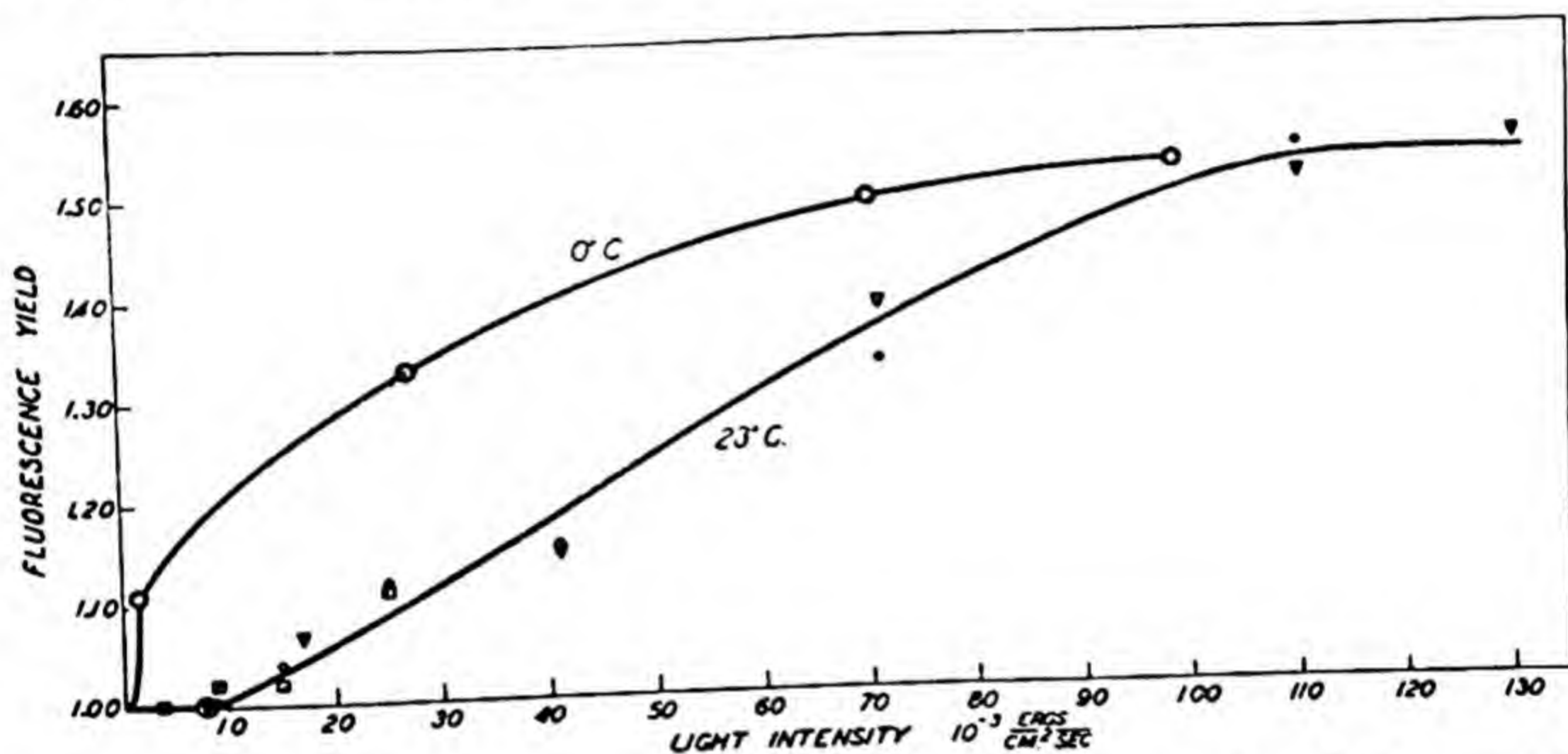


FIG. 16.7—Fluorescence yield as a function of the intensity of the exciting light at room temperature, and at 0°C ., where photosynthesis is inhibited.

one-half to one per cent O_2 no deviation of the fluorescence curve from linearity occurs in the range of light intensities covered by the observation. Franck, French, and Puck (6), who measured the fluorescence curve in leaves of *Hydrangea* in an atmosphere of 97 per cent N_2 , 2 per cent O_2 , and 1 per cent CO_2 , found a deviation from linearity only at light intensities higher than the ones used by McAlister and Myers. Thus the results of McAlister and Myers and those of the Chicago group are in accord.

There is one case in which fluorescence intensity rises perfectly linearly with the intensity of the exciting light. This is observed when a narcotic substance, such as urethane, which almost completely inhibits photosynthesis, is added. The fluorescence yield is constant at all intensities but is two to four times higher than in the absence of the narcotic. This influence of narcotics on the fluorescence yield was first observed by the Dutch group. According to the generally accepted interpretation of Warburg, these

narcotic substances are adsorbed on the surface of the chlorophyll-protein complex and, if they cover the whole surface, prevent all photochemical activity.

If we accept as valid, for the conditions in plants, the results of experiments with chlorophyll and other dyes *in vitro*, we can explain the strong enhancement of the fluorescence by narcotics as a prolongation of the time needed to reach the critical point for

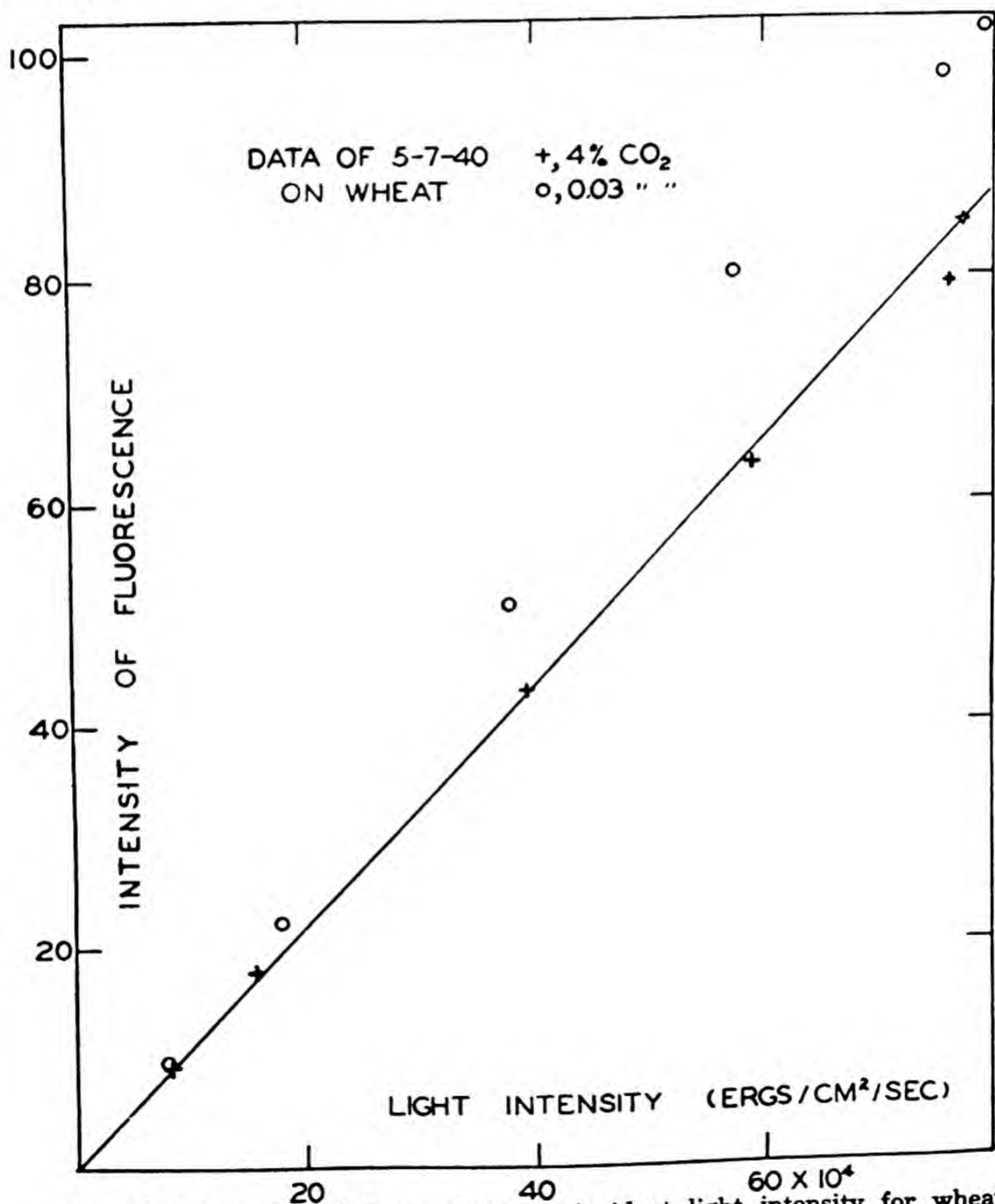


FIG. 16.8—Intensity of fluorescence vs. incident light intensity for wheat at 4 per cent and at 0.03 per cent CO₂.

a radiationless transition. This prolongation would be the result of a decrease in the mobility of the atomic constituents of the chlorophyll in analogy to the enhancement of fluorescence by adsorption of a dye on surfaces (cf. Introduction).

The experiments discussed above give clear evidence that whenever saturation is entirely or partly determined by the rate of formation of CO_2 complexes the fluorescence yield will be greater

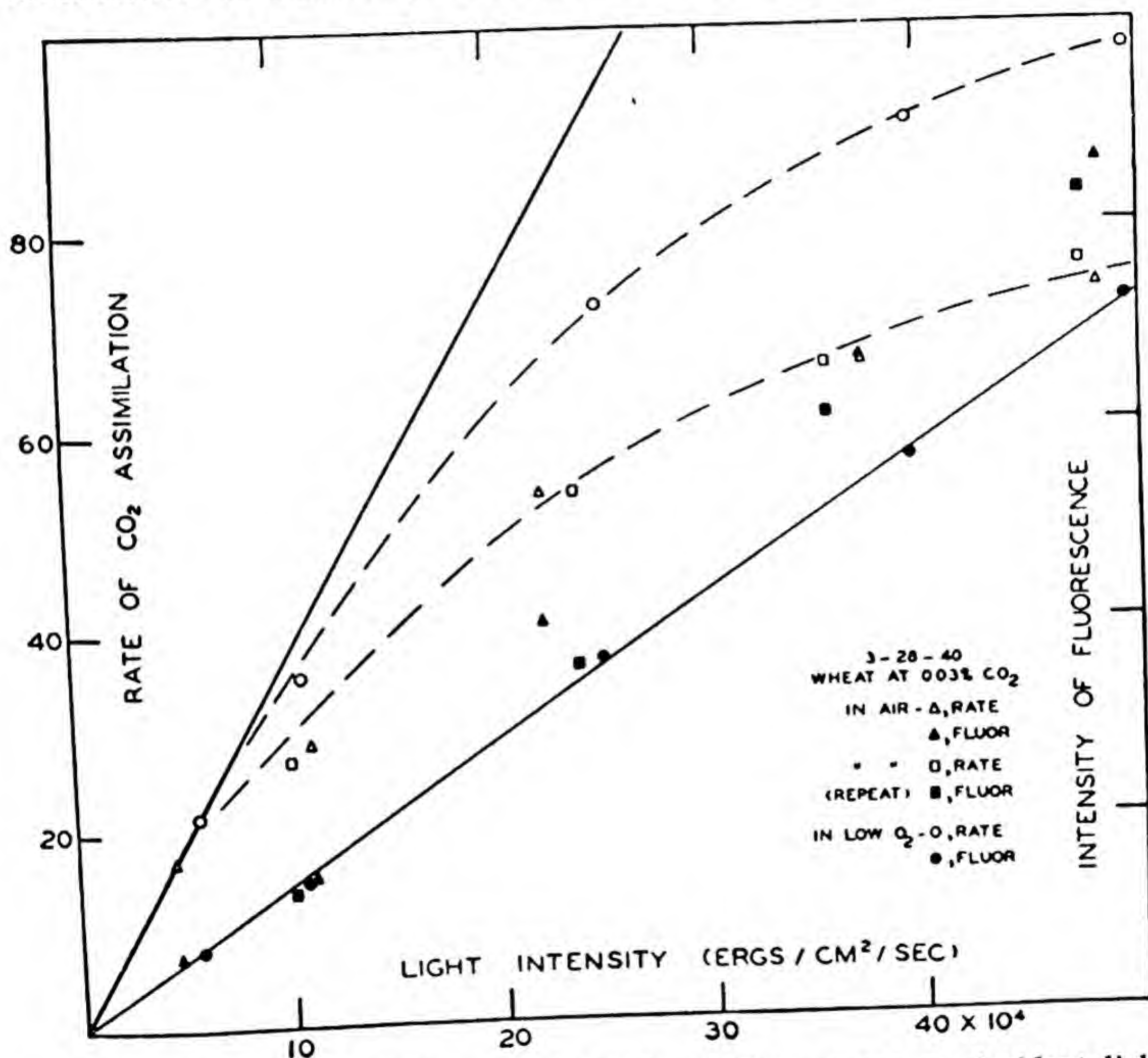


FIG. 16.9—CO₂ assimilation and intensity of fluorescence *vs.* incident light intensity. A comparison of low and normal oxygen pressures for wheat at 0.03 per cent CO₂.

at saturation than it would be if some other factor were limiting. Consequently, the results of measurements of the chlorophyll fluorescence give no reason whatsoever to reject the assumption that CO_2 is reduced directly in contact with the chlorophyll.

However, there are two possible ways in which to interpret the

increase in the fluorescence yield caused by a lack of CO_2 complexes at the surface of the chlorophyll. The enhancement of the yield is either caused directly by denudation of the chlorophyll surface through removal of energy acceptors, or some other substance is absorbed on the chlorophyll-protein surface, and by its adsorption raises the fluorescence yield in a manner similar to the action of a narcotic. This latter hypothesis was first put forward by Franck, French, and Puck (6) to explain the fluorescence anomalies observed during the induction period (cf. Short Induction Period). As more data became available on fluorescence yield measurements with plants in their steady state of the fluorescence, the writer became convinced that all large changes in the fluorescence yield are caused by natural narcotics in plants. Information concerning the metabolic process by which they are produced in plants and about their chemical nature has to be taken from other types of observations which are discussed in the following pages.

PHOTOOXIDATION AND ITS INFLUENCE ON THE STEADY STATE OF FLUORESCENCE IN GREEN PLANTS

The most important photochemical reaction which can be sensitized by chlorophyll *in vitro* as well as in plants is photooxidation involving molecular oxygen. Why then does this dangerous process of photooxidation not destroy the plants immediately, even when only a part of the chlorophyll surface is available for that process? Indeed, it is one of the miracles of photosynthesis that the plant can use a dye able to fluoresce in the presence of oxygen predominately for the purpose of reduction, and is able to hold the process of photooxidation in check so that damage is prevented or minimized even under severe conditions.

The occurrence of photooxidation in plants was first observed directly by Noack. He introduced easily oxidizable substances, which changed their color by oxidation, into the plants and visually observed their photooxidation. He and others have shown that the rate of photooxidation rises if CO_2 limitations are imposed on the plants. As long as photooxidation is not excessive it does no damage to plants in which sufficient carbohydrates are present.

Photooxidation sensitized by chlorophyll dissolved in organic solvents was mentioned in the introduction. But for comparison with conditions existing in plants, observations of photooxidation made by Gaffron are of greater significance. They were carried out with the sensitizing chlorophyll (or porphyrin) adsorbed on serum protein suspended in an aqueous solution. It is known that under these conditions the chlorophyll is able to fluoresce with a small yield, probably of the same order of magnitude as the fluorescence yield in plants. The influence of oxygen on the fluorescence

intensity was not studied. If it had any influence it could only act as a quencher of the fluorescence. The most significant result obtained with this system was the observation that the rate of photooxidation depended upon the concentration of oxygen dissolved in the water. It was shown to be the same in this system as the dependence of the depression by oxygen of photosynthetic rates at saturation observed by Warburg.

Myers and Burr considered that the depression of photosynthetic rates by oxygen was too great to be directly caused by photooxidation. They concluded that an inhibitor of photosynthesis is produced by photooxidation. Franck and French came to the same conclusion. The latter investigators measured the oxygen uptake due to photooxidation in living and dead leaves (boiled for 30 sec.) and compared this oxygen consumption with the depression of the photosynthetic rates. They found that the consumption of O_2 by photooxidation is ten to twenty times smaller than the depression of the photosynthetic oxygen production.

We now want to apply these results to the explanation of the rise of the fluorescence yield observed in living plants when CO_2 complexes are lacking. Under this condition the surface of the chlorophyll-protein complex will not stay photochemically idle but photooxidation will occur. Either no change or, more probably, a decrease in the fluorescence yield will result from direct action of the oxygen on light-excited chlorophyll. Since, however, an increase occurs, we conclude that some substance, made by photooxidation, is adsorbed on the partially denuded chlorophyll-protein surface and causes the fluorescence yield to rise. A substance which combines this property with the one of inhibiting the photosynthetic rates must be a narcotic.

While no direct information is at hand concerning the chemical nature of the narcotic-acting substance, a guess that it may be some plant acid can be based on the following arguments: The presence of carbohydrates is known to offer some protection to the plant against attack by photooxidation on vital components of the cell. This indicates, in agreement with general information about photooxidation *in vitro*, that easily oxidizable substances, like reducing sugars, are the substances preferentially attacked by photooxidation. Since (as will be discussed in more detail in the next section) the natural narcotic is removable by further oxidation, we suggest that it is an intermediate oxidation product of carbohydrate, i.e., a plant acid. It is not necessary to assume that the compound is directly adsorbed on the chlorophyll. It can just as well be that adsorption at other groups of the chlorophyll-protein complex, for instance at the protein, may be responsible for the narcotic action. Because it is a process in which no participation of enzymes is

involved, the formation of a narcotic layer is actually the only way by which the plant can protect itself against excessive and damaging photooxidation. Photooxidation cannot be inhibited by a substance which poisons enzymes but only by one which prevents photochemical activity of the dye.

The explanation that the greater increase in fluorescence yield is not directly caused by lack of CO_2 complexes but rather by photooxidation, favored when the competition of the CO_2 complexes for the excitation energy of the chlorophyll becomes small because of their low concentration, is borne out by McAlister and Myers. They observe (Fig. 16.9) that no rise in fluorescence intensity occurs if the fluorescence curve is measured at moderate light intensities in an atmosphere of nitrogen containing only ~ 1 per cent of oxygen. The observation of Franck, French, and Puck of an increase in the fluorescence yield at very high light intensities in leaves surrounded by an atmosphere of nitrogen containing 2 per cent O_2 and 1 per cent CO_2 (not sufficient to avoid all CO_2 limitations) is also explained. Photooxidation continues to rise with the intensity of the exciting light even in the intensity region of light saturation of photosynthesis. Thus the formation of narcotic, though small in an atmosphere containing only 2 per cent of O_2 , becomes observable if a very high intensity of light is used.

The only experimental fact not covered by the interpretations given so far is the non-linearity of the fluorescence curve in nitrogen carefully freed of all oxygen. An explanation will be given in the next section in which evidence will be presented that a narcotic substance can be made not only by oxidation but also by the process of fermentation in the absence of oxygen.

TRANSIENT PHENOMENA OF THE PHOTOSYNTHETIC RATES AND THE FLUORESCENCE INTENSITY OF CHLOROPHYLL IN PLANTS

Even if the external conditions are kept perfectly constant, neither the photosynthetic rate nor the fluorescence intensity of the chlorophyll stays constant in plants throughout an irradiation period. At the beginning of an irradiation period or when the light intensity is suddenly increased, irregularities occur; the fluorescence intensity becomes considerably higher than the steady state fluorescence, while the photosynthetic rate is depressed during this period. One type of irregularity has a duration which depends upon the intensity of the exciting light and, under suitable conditions, it may take a long time before the normal steady state is reached. This is called the long induction period. Another type lasts about one minute, is independent of the exciting light intensity, and is called the normal or short induction period. A third type of slow rise and

fall of the fluorescence yield and corresponding depression and recovery of the photosynthetic rates is observed with higher plants during prolonged irradiation periods. It belongs to the class of light-induced limitations of photosynthesis.

LONG INDUCTION PERIOD

The long induction period is caused by an inhibition which affects photoreduction as well as photosynthesis. The evidence indicates that this inhibitor is a natural narcotic produced by fermentation. In Figures 16.10 and 16.11 examples of the dependence of fluorescence intensity upon duration of irradiation are plotted for *Chlorella* and *Scenedesmus* in air and in O_2 -free nitrogen. Figure 16.10 shows the very first seconds of the fluorescence time curves, and Figure 16.11 the course of the curves during several minutes until a steady state is reached. The differences between the curves for *Scenedesmus* and *Chlorella* are typical for these particular genera of alga in so far that, if treated alike, they usually show these differences. However, previous history, age of culture, etc., can produce greater differences than the ones present in the curves. For instance, in both algae the starting point becomes systematically higher, the first rise less pronounced, and the decay slower if the duration of the preceding period of anaerobicity is prolonged. The discussion of the rapid rise and fall of the intensities which are connected with the short induction period will be postponed. We shall focus our interest on the difference in the starting points of the curves taken in nitrogen and in air and on the prolongation of the decay to the steady state caused by absence of oxygen. The dotted lines indicate that if the oxygen made photosynthetically is permitted to accumulate, the fluorescence intensity continues to drop slowly to the level of the air curve or, under some conditions, even somewhat lower.

The general shape of these curves was first observed by Kautsky and co-workers and, in much more detail, by Katz and Wassink. The curves reproduced are, however, taken from a paper of Shiau and Franck (8) because these measurements have the advantage in that the starting point of the fluorescence intensity can be precisely determined, and the accumulation of oxygen in the nitrogen can be permitted or prevented at will. Katz and Wassink assume that the entire difference between the curves taken in O_2 -free nitrogen and in air is due to the quenching of the fluorescence of chlorophyll by oxygen. This assumption is part of a theory of photosynthesis advanced by Kautsky and co-workers which Katz and Wassink otherwise reject. It is a peculiar kind of quenching which they are forced to assume. At very low oxygen

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concentration, oxygen is supposed to be a very efficient quencher, but as soon as its partial pressure in the gas phase is of the order of 1 per cent of an atmosphere, it becomes very inefficient. Practically no further quenching is assumed to occur if the partial pressure in the gas phase is raised from 1 per cent to 100 per cent.

Katz and Wassink's assumption that oxygen takes away from the chlorophyll energy which would otherwise be used for photosynthesis makes them expect that the photosynthetic rate in O_2 -free nitrogen at the very start of their measurements must be higher than it is later, when the accumulated oxygen competes strongly with photosynthesis for the use of the excitation energy. But actually the opposite is true, as may be seen from time curves of oxygen production (Figs. 16.12 and 16.13) measured by Franck, Pringsheim, and Lad (7) under the same conditions as the fluorescence curves of Figures 16.10 and 16.11. Oxygen-free nitrogen bubbling through the algal suspension kept the concentration of photosynthetic oxygen exceedingly low. The time lag between the initial and end point of the illumination period relative to the corresponding points of the oxygen production is irrelevant, as it is only a measure of the time needed for the gas stream to flow from the glass tube in which the algae are contained to that part of the apparatus where the oxygen concentration is measured. In the curve of Figure 16.12, measured with *Scenedesmus*, the rate rises as rapidly as the fluorescence decays in the corresponding curve of Figure 16.10b. The curve of Figure 16.13 measured with *Chlorella* shows, on the other hand, a slow rise of the photosynthetic rate just as the corresponding fluorescence curve of Figure 16.10a would lead us to expect.

Willstätter, Noack, and Gaffron much earlier observed that, after a period of anaerobicity, very long induction periods occur which are easily measurable even with the relatively slow manometric methods. Under extreme conditions, in high cell concentration and very long duration of anaerobicity, photosynthesis may not start at all. However, as Noack and co-workers have shown, photosynthetic activity is quite rapidly resumed if oxygen is admitted or if the solution in which the algae are suspended is made alkaline.

All of these results show that during prolonged anaerobicity an inhibitor is formed, and that extreme conditions can cause the formation of enough to totally inhibit the process of photosynthesis. If, on the other hand, only a strong but not a total inhibition is reached, photosynthesis starts autocatalytically and continues until oxygen accumulates in concentration of ~ 1 per cent of the gas atmosphere which is enough to remove all traces of the inhibition. From the fact that this particular inhibitor vanishes just at the partial pressure of oxygen necessary to replace fermentation by respiration, we conclude

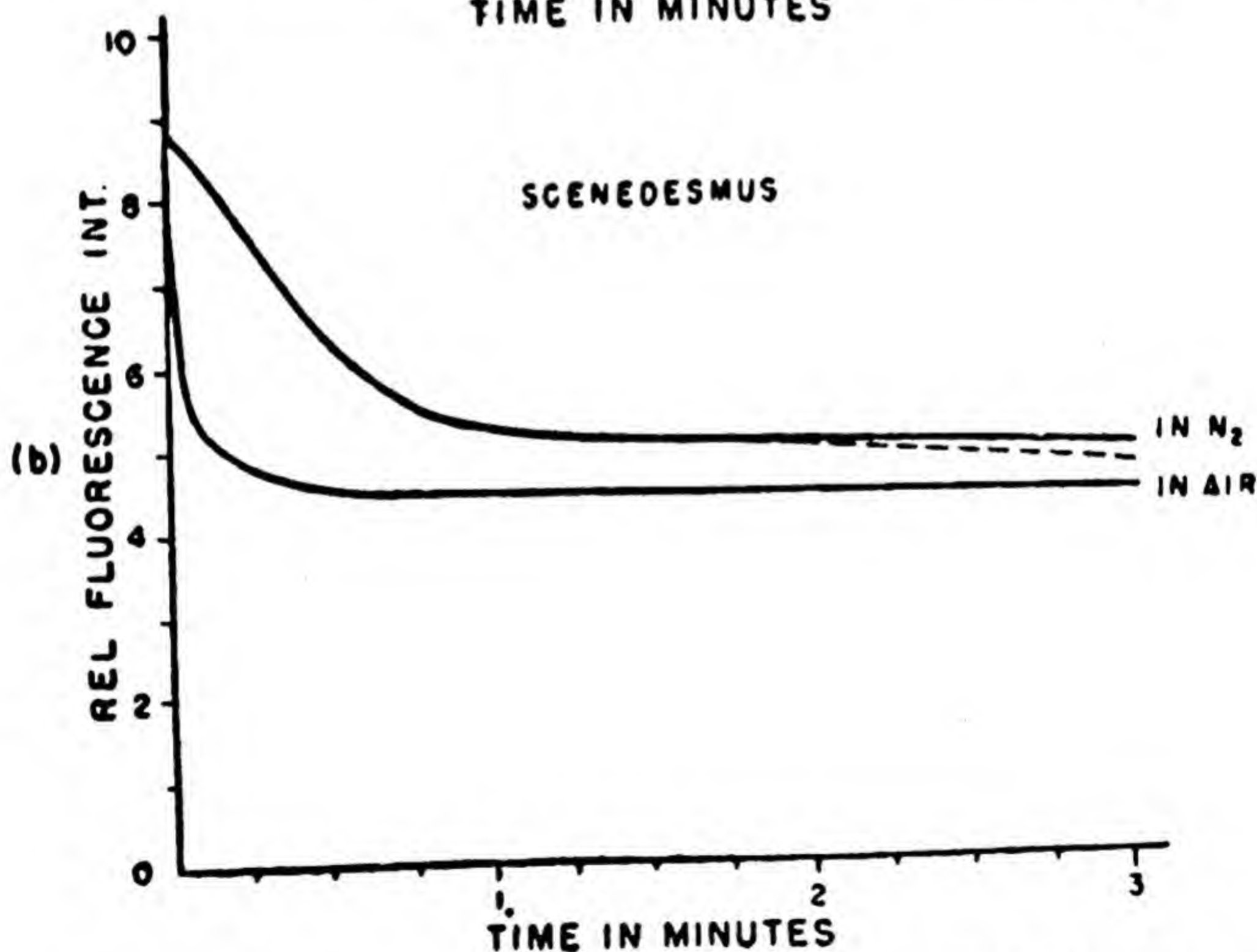
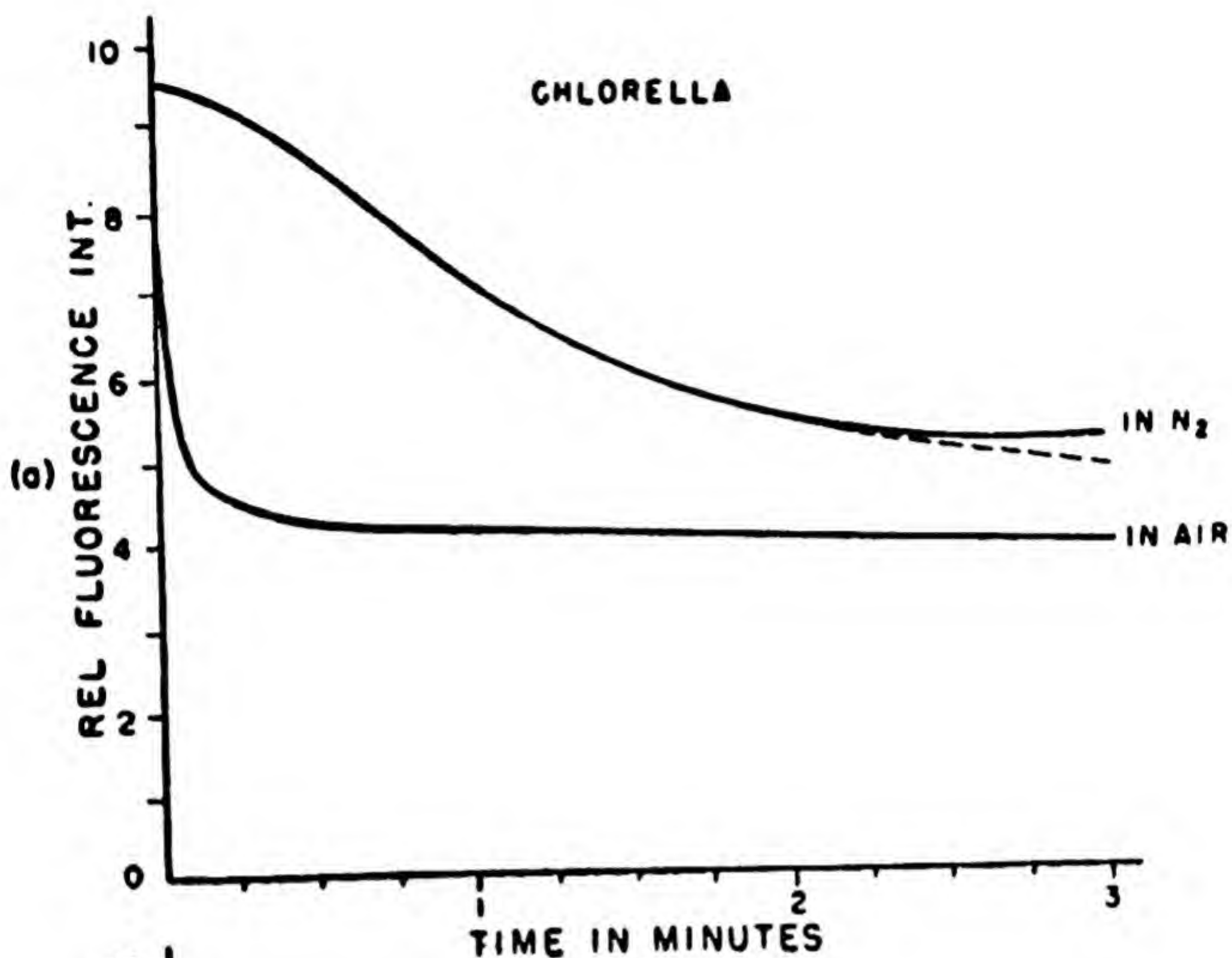


FIG. 16.10—Fluorescence time curves of (a) Chlorella and (b) Scenedesmus, 25°C. and 3.0×10^4 erg/cm.²/sec. incident light intensity, showing the induction phenomena. Relative fluorescence intensities are expressed in arbitrary units which differ for (a) and (b).

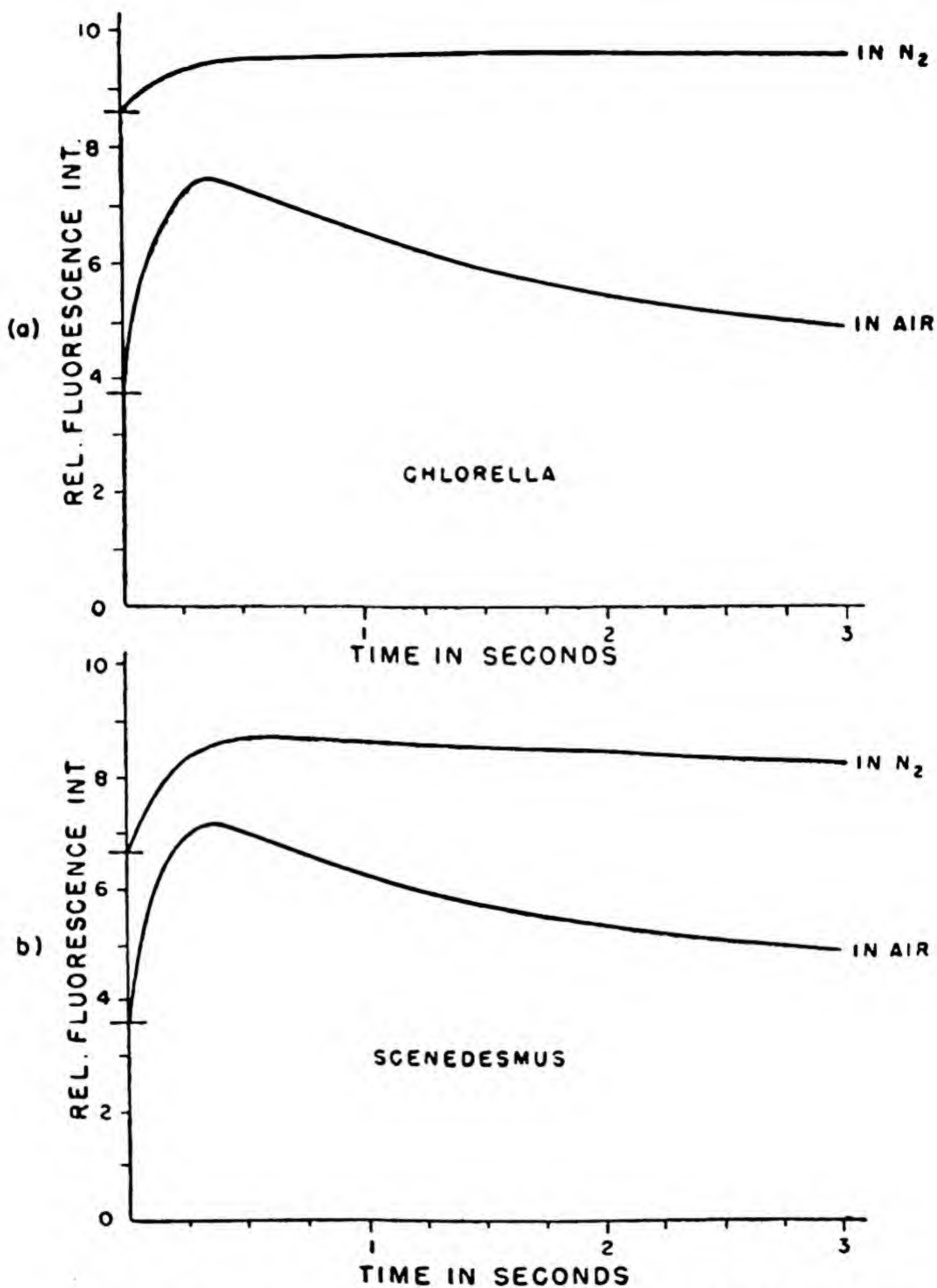


FIG. 16.11—Fluorescence time curves of (a) *Chlorella* and (b) *Scenedesmus* at 25°C. and 3.0×10^4 erg/cm²/sec., showing the initial rises.

that it is an intermediate product of fermentation. Since the inhibitor raises the fluorescence yield at all intensities, we conclude that it has the properties of a narcotic. Noack's observation that the inhibition can be removed by alkalinity is an indication that it is a plant acid. Neutralization of the acid removes the layer from the chlorophyll surface and consequently the fluorescence intensity should drop to its normal value. As Shiau and Franck observed, that is

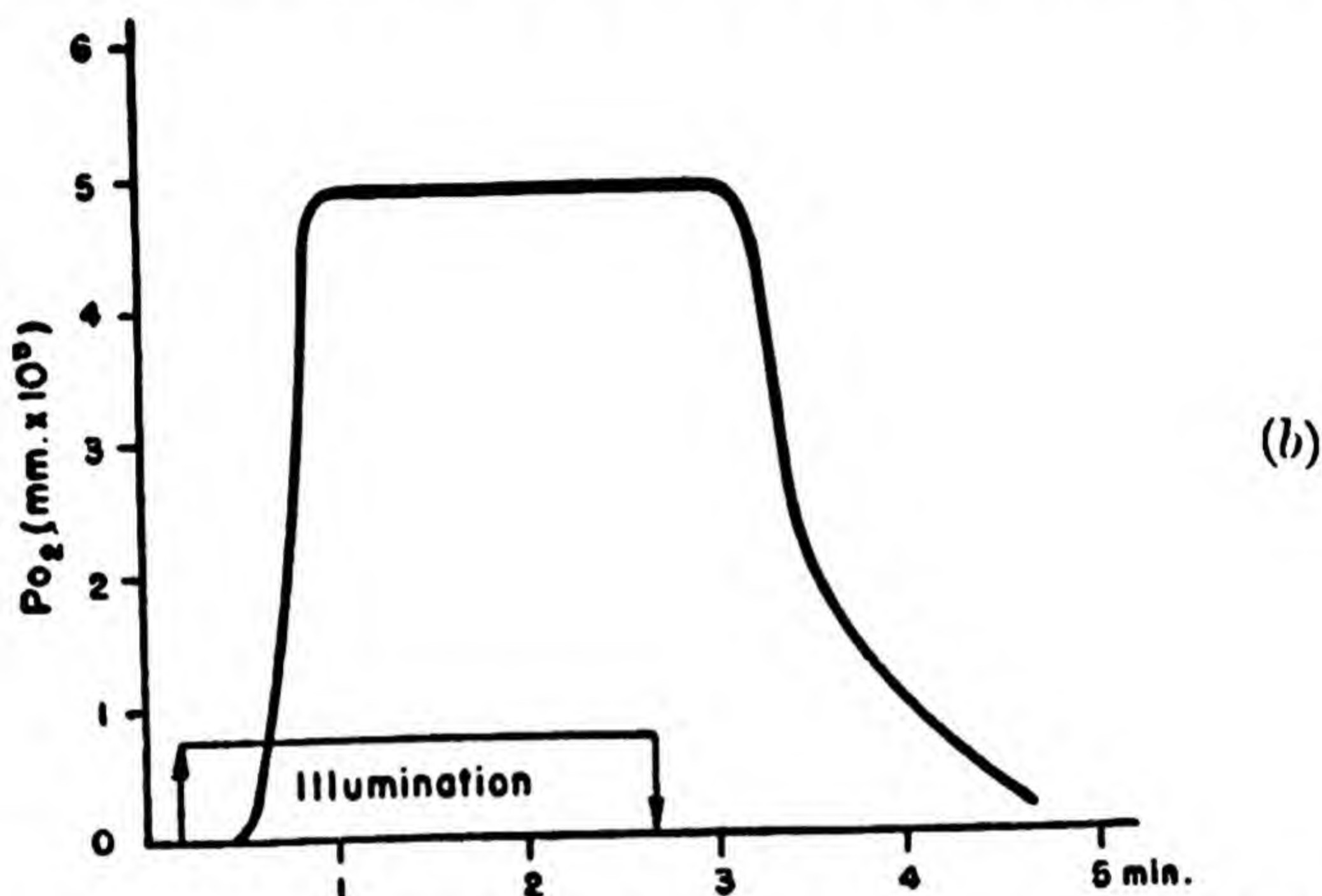


FIG. 16.12—Time curve of oxygen production during illumination of 5×10^{-3} g. *Scenedesmus* in $2\frac{1}{2}$ ml. solution after 3 hours anaerobicity in nitrogen.

indeed the case. Addition of alkali after twelve to twenty-four hours of anaerobicity decreases the fluorescence intensity in algae and in chloroplasts, and no new rise of the fluorescence intensity can be achieved by prolonged dark periods under anaerobic conditions.

The narcotic substance made by fermentation is destroyed not only by the process of respiration but also by other oxidation processes. The shape of the curve in Figure 16.10 indicates that the narcotic layer is partially removed by illumination, even if molecular oxygen is so quickly carried away by a stream of nitrogen that respiration is negligible. Since, under the conditions of the experiment, the surface layer is not sufficiently developed to prevent all photochemical activity, photoperoxides are made as a part of the process of photosynthesis. These photoperoxides, not being quickly removed because the oxygen liberating enzyme is inactivated by anaerobicity, are able to oxidize the narcotic to CO_2 and

water. Since the production by fermentation is a very slow process (as is indicated by the fact that very long, dark periods are necessary to return the fluorescence yield to the value it had at the start of the first illumination period), the equilibrium concentration of the narcotic becomes considerably lower in the light than in the dark. This interpretation is supported by results of measurements of the saturation curves of photosynthesis under anaerobic conditions.

If photoperoxides are able to remove the narcotic made by fermentation, the inhibition caused by the narcotic should be more

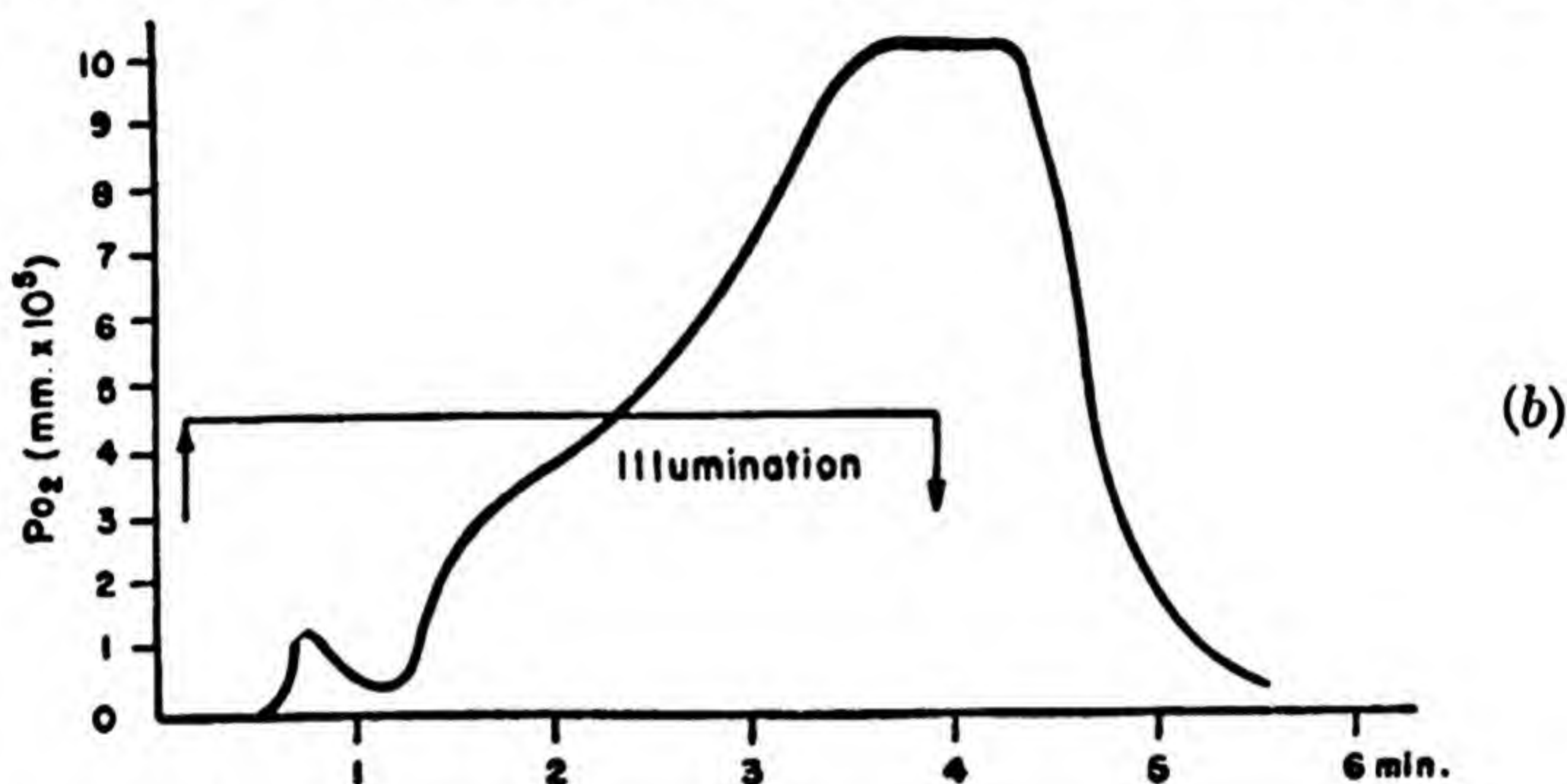


FIG. 16.13—Time curve of oxygen production during illumination of 7.5×10^{-4} g. *Chlorella* in $2\frac{1}{2}$ ml. solution after 3 hours anaerobicity in nitrogen.

pronounced at low light intensities than at higher ones. This will produce a sigmoid shape in the saturation curves. Figure 16.14, taken from observations of Franck, Pringsheim, and Lad (7), may serve as an example which shows the expected effect. The longer the duration of anaerobic pre-treatment, the more pronounced becomes the sigmoid shape. Saturation curves of the same sigmoidal shape (only much less pronounced) are always observed in the anaerobic process of photoreduction with *Scenedesmus* and with purple bacteria. We believe that a gradual removal of a narcotic with rising light intensity may also be partly responsible for the S-shape in these cases. This question will be included in our discussion of purple bacteria.

SHORT INDUCTION PERIOD

The short induction period is a transient depression of the photosynthetic rate occurring at the beginning of an illumination period. Simultaneously, the fluorescence yield is raised anti-parallel to the depression of the photosynthetic rate. Figure 16.15 gives an example,

typical for these phenomena, observed with leaves of higher plants. The curves represent the photosynthetic rates and the fluorescence intensities plotted against time. The curve of the fluorescence intensities starts with the same value that, under the conditions of the experiment, is reached again at the steady state. During approximately the first half second the curve rises steeply to a maximum, and then goes down again with a smaller slope until, after one minute, the steady state is reached. The photosynthetic rate cannot be measured exactly at the very first moment of the irradiation

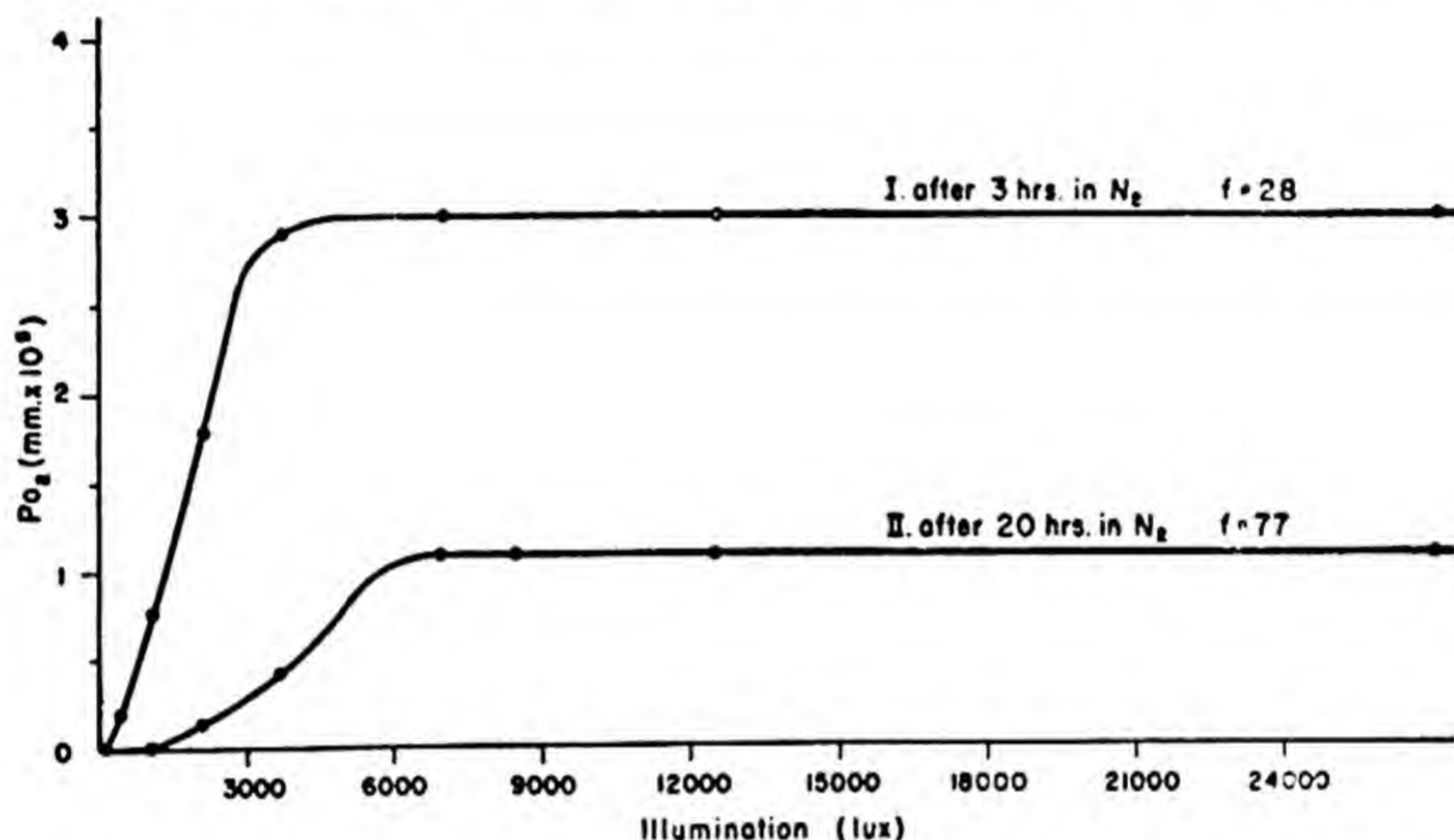


FIG. 16.14—Light saturation curves for oxygen production by 2.5×10^{-5} g. Chlorella in $2\frac{1}{2}$ ml. solution (f designates reduction factor from normal aerobic saturation rate).

period, but the experiments of Blinks and Skow (who used the formation of an oxygen electrode as indicator for the presence of oxygen) unmistakably show that an oxygen evolution occurs during the very first second of irradiation. The initial photosynthetic rate is apparently smaller than the one obtaining after the steady state is reached.

Right from the beginning of the irradiation, photosynthetic rates go down sharply to a very low value and rise again in about one minute to the steady state rate. At very low light intensities anomalies of both the photosynthetic rate and of the fluorescence are absent. At somewhat higher intensities, but still far below the saturation intensity, the induction period is fully developed. This is indicated by the observation that the quotient between the maximum of fluorescence intensity and the value at the steady state remains constant down to quite low intensities of the exciting light. The rate curves show a

corresponding behavior. The correspondence between an increase in the fluorescence yield and a depression of the photosynthetic rate is so perfect, and has been confirmed so often, that in many cases it is sufficient to measure only the exact shape of the fluorescence-time curve, which is much easier to observe than is the exact shape of the photosynthetic rate curve.

If the light intensity is suddenly increased after a steady state

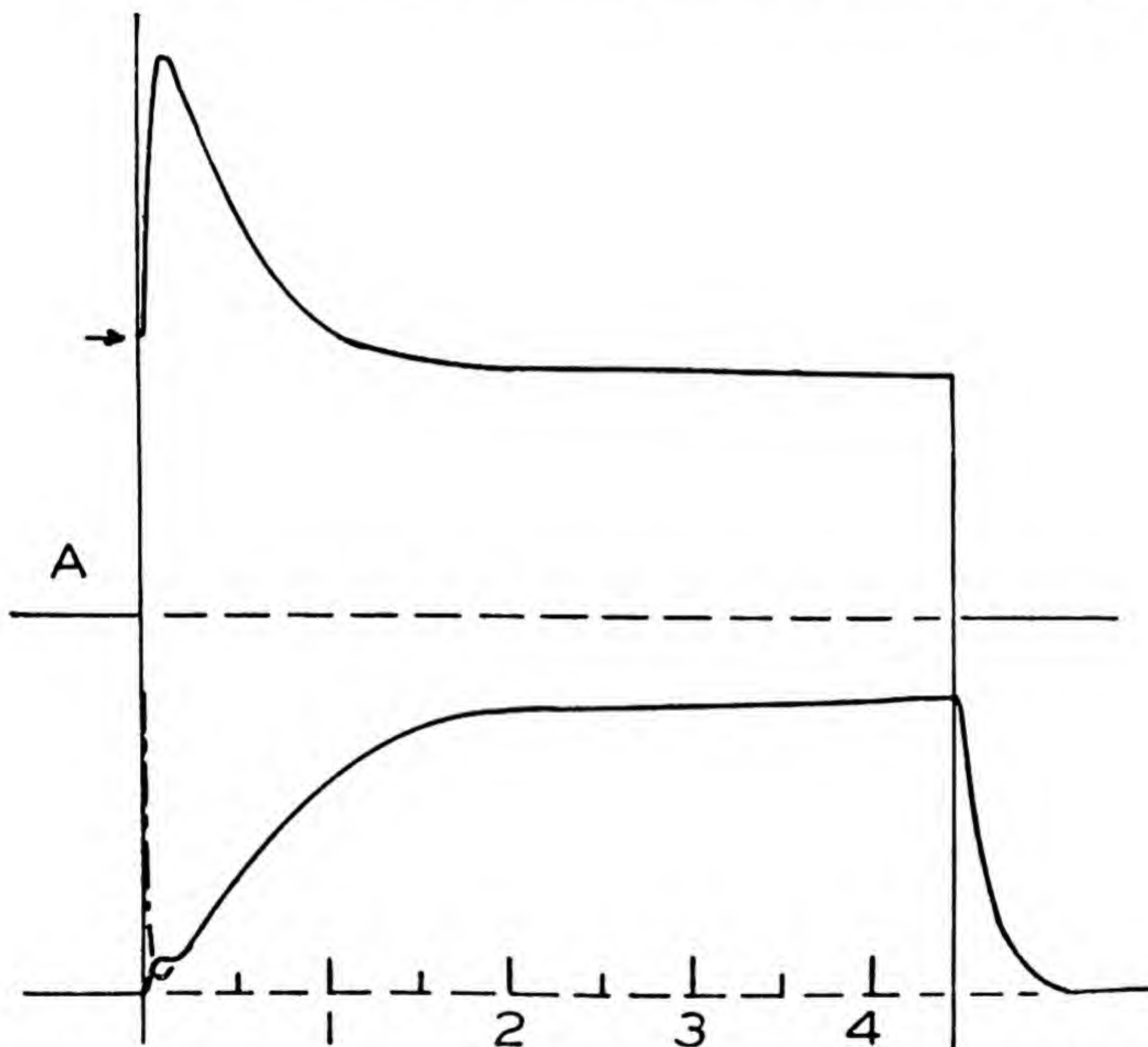


FIG. 16.15—Induction behavior of wheat at low oxygen pressure, 0.03 per cent CO_2 , high light intensity after 30-minute dark rest.

has been reached during irradiation with low or medium intensities, measurements of the photosynthetic rate and of the fluorescence yield curves show that the induction phenomenon re-occurs. This means that the transition from a lower rate of photosynthesis to a higher one involves an initial depression of the rate, followed by a rise to the new higher values. Similar phenomena occur if the transition to a higher photosynthetic rate is induced by a sudden

increase in CO_2 concentration. In this case, however, a brief depression of the fluorescence intensity is observed in the first second after addition of CO_2 to the gas atmosphere. This is followed by the fluorescence rise and fall typical for the induction period. The initial dip in the fluorescence curve is an indication that the increased concentration of CO_2 complexes has displaced some narcotic from the chlorophyll.

The observations mentioned so far are sufficient to show that the induction period is not comparable to the usual photochemical induction periods caused by the necessity for photochemical changes to occur in the substrate before the reaction under observation can proceed normally. If such were the reason for the short induction period of photosynthesis, its duration should be long at low intensities and short at higher ones. However, the duration of this induction period is independent of the exciting-light intensity in leaves measured at normal temperatures in air containing CO_2 at concentrations between 0.04 per cent and 2.0 per cent. Furthermore, the rates should rise monotonously from a low value in the beginning to the steady state.

The observation that no induction phenomenon occurs at very low light intensities while it is present at higher ones indicates that it is due to an inactivation during the preceding dark period of one of the enzymes involved in photosynthesis. Three different enzymes are known to influence the photosynthetic rates: one which combines the CO_2 with another molecule, probably in a carboxylation reaction, before the photochemical steps take place; a second which transforms unstable photochemical products into stable ones; and a third which liberates the oxygen from the so-called photoperoxides.

The inactivation of the first of these enzymes cannot be responsible for the induction phenomenon for these reasons: Cyanide and low temperature slow down the rate of formation of CO_2 complexes, but the time course of the photosynthetic rate (7), and of the fluorescence yield (8) during the first part of the irradiation period are exactly the same whether cyanide is present or not. Figure 16.6b, shows that in air the time course of fluorescence is the same for approximately twenty seconds whether cyanide is present or not, and that in pure nitrogen it is the same during the first $1\frac{1}{2}$ minutes of irradiation. Limitations on the enzymatic formation of CO_2 complexes cannot, therefore, be responsible for the occurrence of the induction.

Neither can inactivation of the second enzyme be responsible for the induction period because the unstable photoproducts, not stabilized quickly enough by action of the enzyme, fall back into

the original chemical state. Therefore, even if the enzyme is fully deactivated, the concentration of photosensitive CO_2 complexes and intermediates of photosynthesis will remain at the same values present at the very beginning of the irradiation, and no rise in the fluorescence yield can occur.

By exclusion we decide that the cause of the short induction period must be the deactivation of the third enzyme during the dark

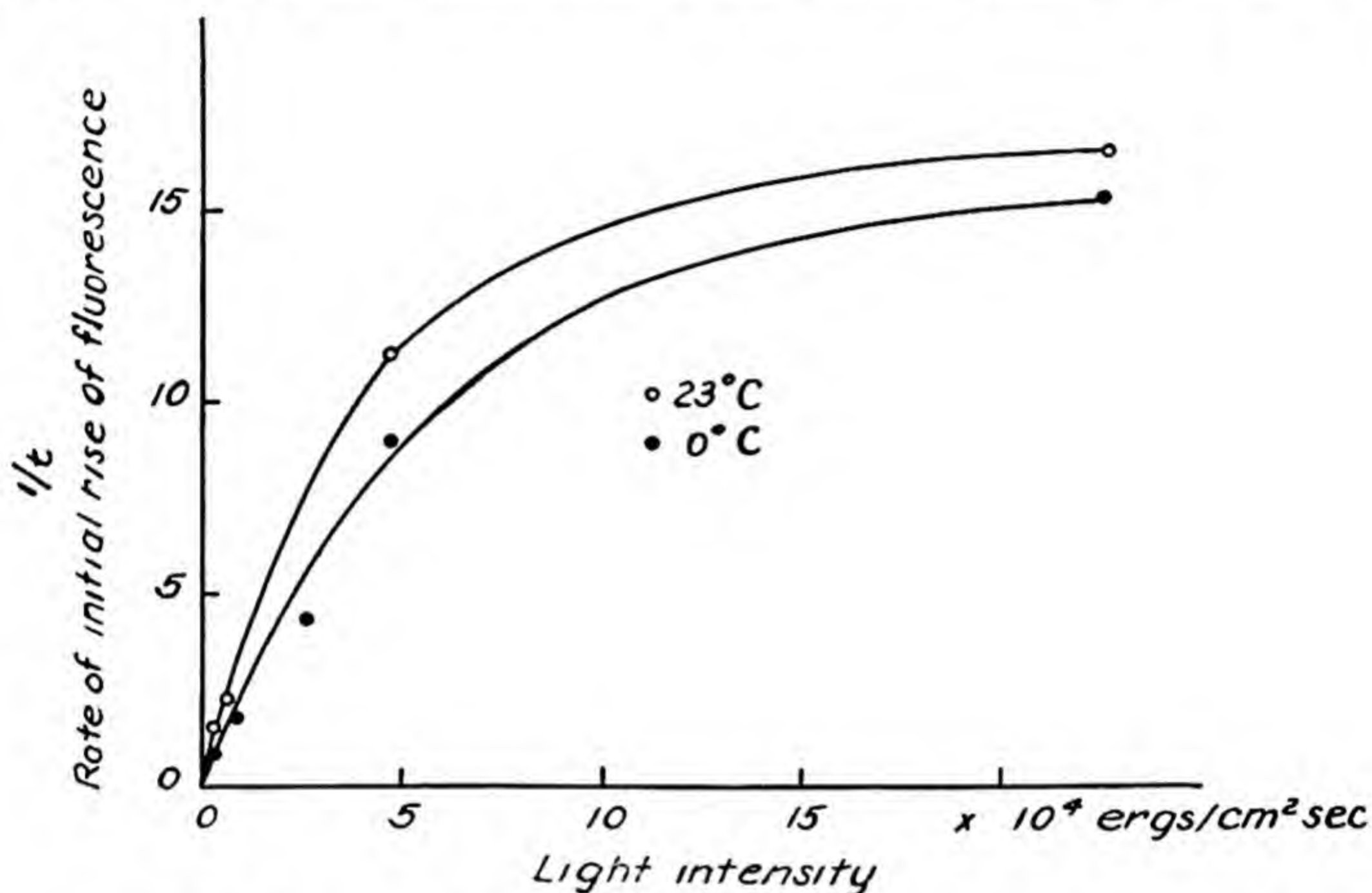


FIG. 16.16—The rate of the initial rise of fluorescence (as measured by the reciprocal of the time necessary for attainment of one-half the maximum intensity) increases with light intensity in the same fashion as does the rate of photosynthesis.

period. If the other enzymes remain active, the photosynthetic production of photoperoxides will start at a normal rate. With the O_2 -liberating enzyme deactivated, the photoperoxides will attack easily oxidizable substances and, just as in the process of photooxidation, form an intermediate oxidation product with the properties of a narcotic which will cover the chlorophyll. Thereby the rate of production of photoperoxides will be reduced to such an extent that the residue of still active enzyme, can take care of the small quantity produced and check a further extension of the narcotic layer. The steep drop in the rate of O_2 production in the first second of irradiation and the correspondingly steep rise in the fluorescence yield is then the result of the formation of the narcotic layer.

The subsequent slow rise of the photosynthetic rate and the slow decline of the fluorescence yield occur during the period in which the enzyme is reactivated and the layer is removed. Since, according to this interpretation, the layer is formed by the action of photosynthetically produced photoperoxides, the rate of its formation; i.e., the initial ascending slope of the fluorescence curve, should show the same dependence upon light intensity as the photosynthetic rates during the steady state. That is, indeed, the case (cf. Fig. 16.16). The steepness of the initial slope of the fluorescence curve increases linearly with an increase in the intensity of the exciting light at lower intensities of the latter, and finally becomes constant. The shape of the rate curve for the production of the narcotic agrees quite well with the normal saturation curve of photosynthesis. That is, however, only true for room temperature.

Lowering of the temperature has much less influence on the initial rate of the layer formation than on the saturation rate of photosynthesis. Actually this is in agreement with our hypothesis. The lowering of the temperature lowers saturation rates mainly by limitation of the formation of CO_2 complexes. However, this has no influence on the photosynthetic activity at the very beginning of an irradiation period. A supply of CO_2 complexes is accumulated during the dark period sufficient to maintain a normal rate of photosynthesis for about a half minute at high light intensities, or longer at low intensities, even if no new formation of complexes takes place.

The natural narcotic formed by the oxidation reaction may be a plant acid. Consequently (cf. pages 314 and 316), the fluorescence outburst should be absent if the narcotic is prevented from settling down on the surface of the chlorophyll by changing the pH inside of the chloroplasts to alkaline. The membranes of the cell become penetrable to alkali by prolonged anaerobicity, and this permeability is retained for some time under aerobic conditions. This makes it possible to show that, if alkali is present in the cells, no fluorescence outburst occurs at the beginning of irradiation periods, either in air or in pure nitrogen. The fluorescence intensity stays perfectly constant and no anomalies of the fluorescence curve occur to indicate an induction period (8). Under these conditions the photosynthetic oxygen production should rise evenly from a relatively low value at the start to the final value of the steady state. An experiment to test this, although difficult, could probably be carried out by using the method of Blinks and Skow.

The explanation for the occurrence of the short induction phenomenon given so far, is the one presented by Franck, French, and Puck (6). It may help to recapitulate the main points:

(a) The cause of the short induction period at the start of illumination is a deactivation of the oxygen liberating enzyme during dark periods and its reactivation during illumination.

(b) The photoperoxides, too slowly removed by oxygen evolution on account of the deactivation of the enzyme, oxidize easily oxidizable substances and convert them into narcotic acting substances.

(c) These natural narcotics cover the chlorophyll complexes and check thereby their photochemical activity.

(d) The narcotics—most probably plant acids—lose their narcotic quality by further oxidation, mostly by respiration. Therefore they are quickly removed when their production ceases on account of the reactivation of the oxygen liberating enzyme.

In the last years more has been learned about the way in which deactivation and reactivation of the enzyme is achieved. It is done with the help of an oxidizable poison, which is made metabolically and whose concentration is high in the dark and low during illumination. The following four paragraphs contain most of the evidence on which this interpretation is based.

It has often been observed that algal cultures grown for a long time in the same culture solution stop further growth when a certain cell density is reached. Pratt has shown that a water-soluble substance excreted by the cells is responsible for the stoppage of growth. From old cultures Spoehr and co-workers were able to isolate chemical compounds which may be identical with the one whose influence Pratt observed. This water-soluble substance can exert its influence only if it is able to penetrate the cell membranes. Two conditions lead to greater permeability of the cell membranes, age and anaerobicity. Shiau and Franck (8) observed, for instance, that when the culture is old, methylene blue is able to penetrate into living cells, whereas it does not penetrate young cells. Franck, Pringsheim, and Lad (7) found that anaerobic treatment of young as well as of old cultures causes the cell membranes to become penetrable. On the basis of various observations, they concluded that this water-soluble excrete of the algae inactivates only the O_2 liberating enzyme, the other enzymes involved in the process of photosynthesis being unaffected. The most direct indication of this is the fact that photoreduction (in the presence of hydrogen) can proceed with the normal quantum yield of photosynthesis under anaerobic conditions. This process is supposed to use the same system of enzymes as photosynthesis with the exception of the O_2 -liberating enzyme. In fact, this enzyme must be inactivated to make photoreduction possible in green algae.

Evidence that the poison of the O_2 -liberating enzyme is an excretion of the algae comes from the observation that the inactivation of

the enzyme increases with an increase in either the duration of the anaerobic treatment or the cell concentration. For instance, after approximately six hours of anaerobicity no decline in the rate of photosynthetic oxygen liberation is detectable if the number of algae per ml. is so small that no green color is visible to the naked eye. On the other hand, the activity of the enzyme is reduced by a factor of a thousand or more if cells at the concentration normally used for photosynthetic measurements are kept equally long under anaerobic conditions. This evidence that the concentration of the poison depends upon the cell concentration is especially important. While the concentration of the poison and the permeability of the cells decline with aerobicity, both probably remain relatively high, even in air, if the cell density is high, as is the case in multicellular leaves of higher plants and in old cultures of algae. The fact that the photosynthetic rates go down as the density and age of algal cultures increase, as Pratt and others have observed, supports this hypothesis. In such cultures the concentration of the poison in the cells may stay high enough to prevent further growth of the culture. At least, presence of this poison will be one of the factors responsible for the decline of growth by contributing to the influences which lower the saturation rate of photosynthesis as the age and density of cultures becomes greater.

If the oxygen made photosynthetically is quickly removed by bubbling a stream of O_2 -free gas through the algal suspension, the enzyme remains poisoned. If, on the other hand, the photosynthetic oxygen is allowed to accumulate, the enzyme becomes reactivated by removal of the poison until a steady state equilibrium between production and consumption of the inhibitor is reached. The concentration of the poison will, therefore, be lower in the light than it is immediately after a dark period. This explains the autocatalytic reactivation of the O_2 -liberating enzyme during the first minute of irradiation. It is, furthermore, in accordance with the observation that the activity of the enzyme is automatically adapted to the photosynthetic rate, as indicated by the fact that each sudden enhancement of the photosynthetic rate is connected with a new induction period.

A further test of the hypothesis relating the short induction period to poisoning of the O_2 -liberating enzyme may be made by determining the dependence of the phenomenon upon cell concentration. If relatively fresh cultures of algae, in a concentration somewhat smaller than the one usually taken for studies of photosynthesis in Warburg vessels, are used, the induction phenomena observed are much less pronounced and of shorter duration than in leaves. Figures 16.10 and 16.11, used in the previous section for

another purpose, can be taken as examples to show this fact in respect to anomalies in the fluorescence. Shiau and Franck (8) found, furthermore, that in some very young and thin cultures of algae no fluorescence outburst occurred, and concluded, therefore, that no induction period occurred. Otherwise, these algae behaved quite normally and induction phenomena could be induced in them

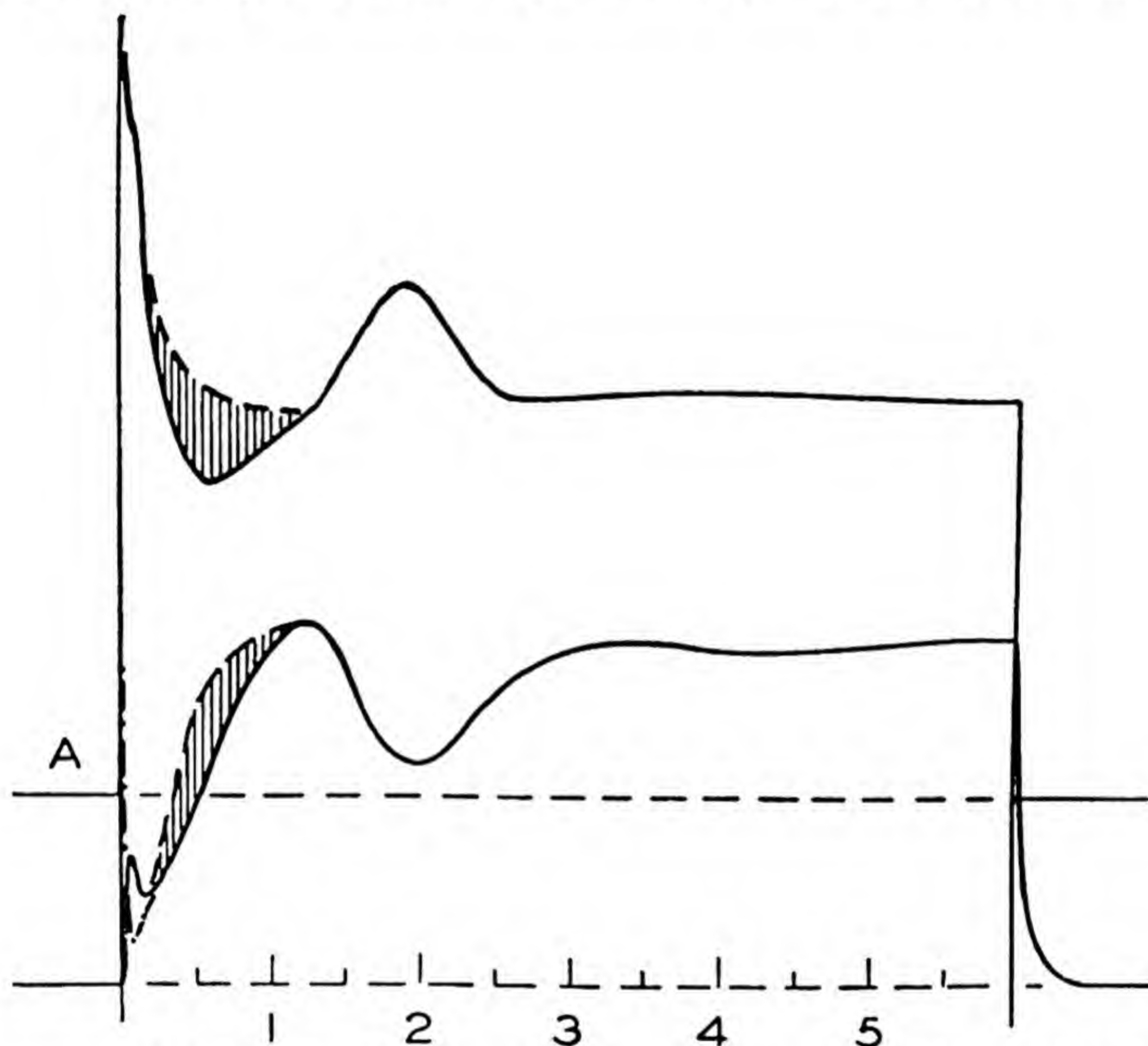


FIG. 16.17—Induction behavior of wheat in 0.24 per cent CO_2 in high light intensity after 30-minute dark rest.

immediately by anaerobicity. Old and dense cultures of algae have, on the other hand, pronounced induction phenomena identical with those observed in leaves. These observations are in strict agreement with the effect under anaerobic conditions of cell concentration upon the activity of the O_2 -liberating enzyme.

Next we have to discuss, that the formation of a narcotic during the induction period is also able to explain the more complicated time courses of the rates of photosynthesis and of the fluorescence intensities which occur under certain conditions. Figure 16.17, taken from observations made by McAlister and Myers with wheat plants

(4), shows an example in which the rate curve of photosynthesis has a second minimum and the curve of fluorescence intensity has a second maximum. Figure 16.18 shows fluorescence-time curves with a second maximum observed with *Chlorella* in air and in pure nitrogen (Shiau and Franck). Figure 16.19 shows a corresponding second minimum for the oxygen production of *Chlorella* under

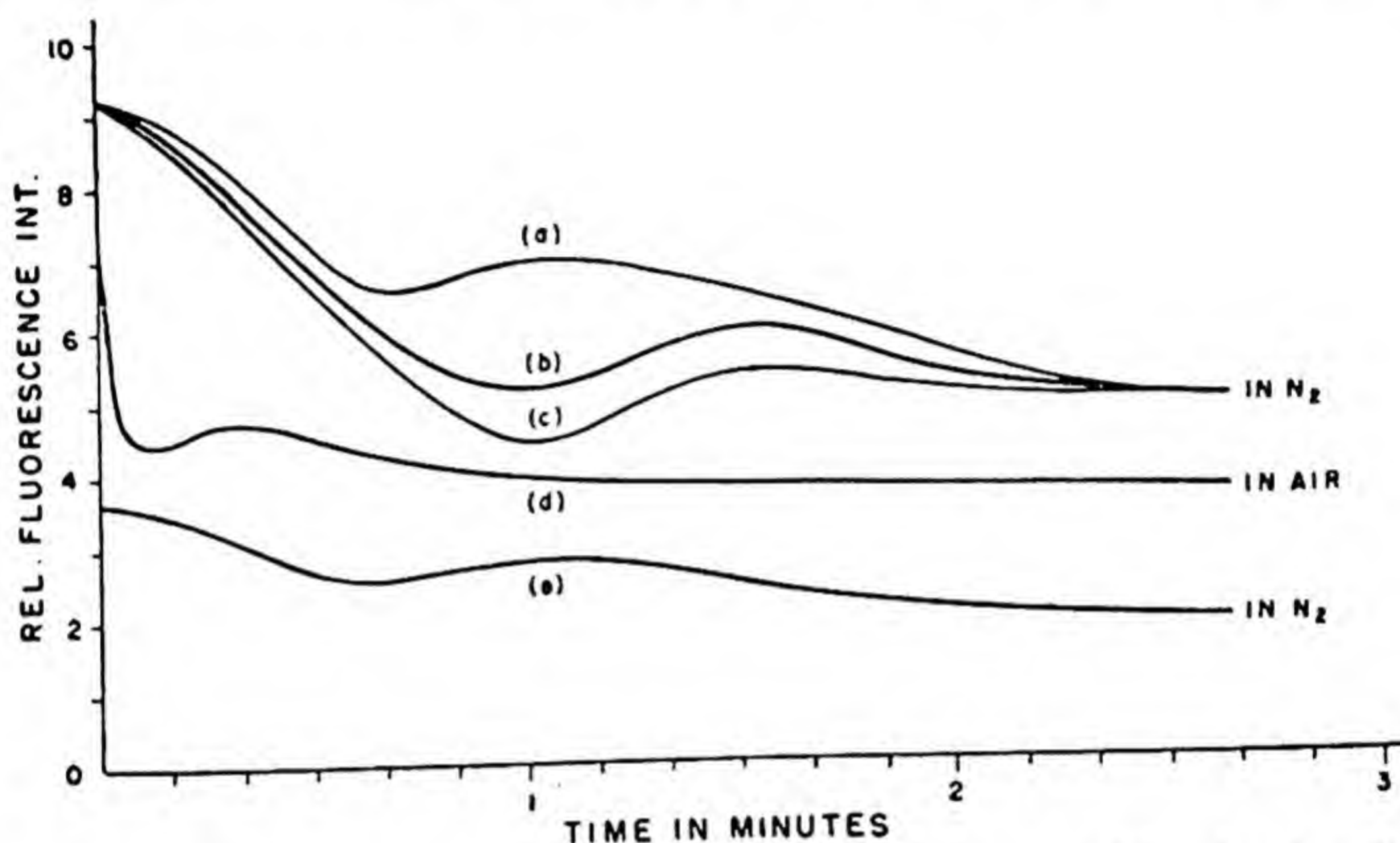


FIG. 16.18—Fluorescence time curves of *Chlorella* at 24°C., showing the secondary induction under different conditions.

Time in darkness:		Gas	Light intensities: erg/cm. ² /sec.
	Hour		
(a)	0.5	N ₂	3.0 x 10 ⁴
(b)	1.0	N ₂	3.0 x 10 ⁴
(c)	2.0	N ₂	3.0 x 10 ⁴
(d)	0.5	Air	3.0 x 10 ⁴
(e)	0.5	N ₂	2.2 x 10 ⁴

anaerobic conditions. These secondary induction phenomena occur only when the rate of formation of CO₂ complexes is the major limiting factor. If the dark period preceding irradiation is of sufficient duration, the concentration of complexes formed in the dark will be considerably higher than the concentration allowed at equilibrium in the light. The transition from the higher to the lower concentration will occur during the first minute of irradiation. Its influence on the fluorescence will thus be superimposed upon the influence of the narcotic which is made and again disappears during the induction period.

Since it is the process of removal of the narcotic whose rate is comparable to the rate of transition from the high to the low concentration of CO₂ complexes, irregularities may be expected

to occur during the time in which both the concentration of the narcotic and of the complexes decline. According to the laws of competitive adsorption, the photosynthetically reducible substances and the narcotic will compete for places on the chlorophyll-protein surface. The percentage of the surface occupied by each of these substances at any moment will depend upon the ratio of their relative concentrations at that particular time. If the concentration of the CO_2 complexes temporarily decreases more rapidly than that of the narcotic, the amount of the narcotic adsorbed on the chlorophyll may rise temporarily, and a secondary maximum of

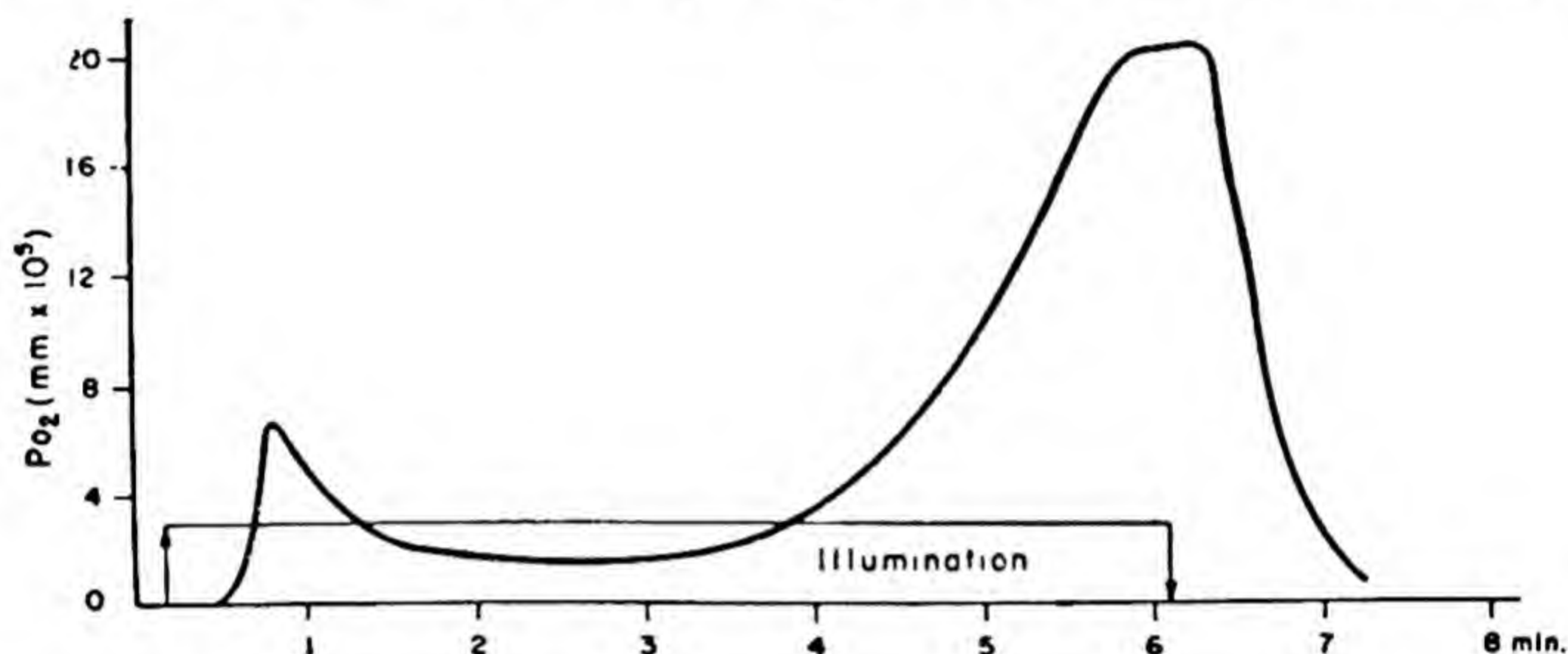


FIG. 16.19—Time curve of oxygen production by 17.5×10^{-3} g. *Chlorella* in $2\frac{1}{2}$ ml. solution at 0°C .

the fluorescence yield result. (A more detailed discussion is given in the paper of Shiau and Franck.) The same considerations apply if the enzyme involved in the formation of CO_2 complexes is poisoned by cyanide to such a degree that the rate of formation of new complexes is practically zero. In that case, the concentration of reducible substances will fall from a high value at the start of the illumination period to a very low one. Consequently, as mentioned before, the oxygen production and the fluorescence yield will not be influenced by cyanide at the beginning of the irradiation period. However, differences in their time courses will develop as soon as the surplus concentration of reducible substances has been reduced to a level where the supply of CO_2 complexes becomes the limiting factor. At this point the fluorescence intensity will rise again and the oxygen production will fall.

Figure 16.6b shows an example of the fluorescence time courses measured under such conditions. The dotted lines represent the path of the curve without cyanide from the point of divergence on. The rise in fluorescence intensity in the presence of cyanide is due in both cases to a covering by narcotic of the chlorophyll-pro-

tein surface which has been denuded of CO_2 complexes. In air the narcotic is produced by photooxidation; in nitrogen it is mostly left over from previous production by fermentation. Similar observations are described in the paper of Franck, Pringsheim, and Lad for the time course of oxygen production in the presence and in the absence of cyanide.

If the CO_2 uptake is limiting, there will also be differences between the rate of O_2 production and of CO_2 uptake during the induction period. The reason is the following: The decomposition of the photoperoxides is a very rapid process. Either they are quickly decomposed by the enzyme or they vanish as quickly by reactions with easily oxidizable substances. There is, therefore, no reason why O_2 production should ever fall out of step with changes in the rate of photochemical formation of the photoperoxides. The situation differs for the formation of CO_2 complexes. When that reaction is limiting, a pickup of CO_2 is observed during interruptions of the illumination. If the limitation is severe, this pickup may continue for about a minute after cessation of the illumination.

This explains McAlister's observation that the time course of the CO_2 uptake is not precisely anti-parallel to the time course of the fluorescence intensity when CO_2 uptake is the limiting factor. For instance, the time courses presented in Figure 16.17 show that the first minimum of the fluorescence does not coincide with the first maximum of CO_2 uptake; the latter is somewhat delayed. Furthermore, the uptake continues for about twenty to thirty seconds after the light is shut off. On the other hand, an exact anti-parallelism should exist between the rate curve of oxygen production and the fluorescence time curve. It is regrettable that none of the methods available for measurement of the time course of oxygen production is accurate enough for a severe test of this prediction. Another consequence of the inertia of the reaction forming CO_2 complexes is that temporary changes in the photosynthetic quotient must occur during the induction period because the CO_2 fixation is unable to follow the relatively quick variations in the photochemical rates.

A host of other observations have been made concerning the fluorescence anomalies observed during the induction period. Measurements have been made of the lifetime of the narcotic substance during dark periods and of the influence of low temperature, high CO_2 concentrations, and addition of cyanide and other poisons on this lifetime and on the duration of the induction period. Other measurements have been made of the time course of fluorescence and of oxygen production in isolated chloroplasts and of the influence on the fluorescence intensity in isolated chloroplasts of quinone and other substances which are reduced by illuminated chloroplasts.

All of these results cannot be discussed in detail; the following resumé will have to suffice.

The narcotic made by oxidation processes has a short lifetime in whole plants or plant cells. It is removed by respiratory processes as well as by oxidation by photoperoxides. This is quite understandable if the narcotic is an intermediate product of the oxidation of carbohydrate, perhaps a plant acid. The fluorescence phenomena in isolated chloroplasts indicate that CO_2 complexes are lacking. If a narcotic layer is formed, for instance, by photooxidation, it has a long lifetime, which is an indication that very little or perhaps no respiration occurs in isolated chloroplasts. The reduction of quinone starts without any induction period and the fluorescence intensity remains constant right from the beginning of irradiation. Its value is about 30 per cent smaller than the steady state fluorescence intensity of chlorophyll in photosynthesizing plants. We regard this as an indication that the relatively simple molecule, quinone, if adsorbed at the chlorophyll, may actually reduce the fluorescence intensity. All of these and other observations are in strict accordance with the assumption that the anomalies observed in the fluorescence during the short induction period are caused by a natural narcotic made from easily oxidizable substances by the action of photoperoxides. The latter is available because of the momentary presence of a poison for the O_2 -liberating enzyme.

LIMITATION OF PHOTOSYNTHESIS INDUCED BY PROLONGED IRRADIATION

There are many observations in the literature which indicate that the photosynthetic activity of higher plants can be checked by internal factors. Harder has shown that the great changes in the rate which sometimes occur after several hours of irradiation are not induced by changes in the external conditions. For instance, after a few hours of exposure to strong light comparable to the intensity of sunlight, plants often reduce their photosynthetic activity to a minimum which may be below the amount which will compensate for respiration. After several hours of rest, the plants resume the original rate of photosynthesis.

It has been shown that this temporary reduction in the photosynthetic activity is not produced by any known morphological change, as for instance, the partial closing of the stomata or movement of the chloroplasts to the so-called systrophic positions. As the plant can produce natural narcotics which, if present in high enough concentrations, can prevent all photochemical action, it is reasonable to suppose that the photosynthetic activity of the plant is regulated by the formation of such narcotics. The reduction of the photosynthetic rate after long exposure to sunshine in the

presence of the amount of CO_2 normally present in the air may be valuable in preventing overfeeding. If this reduction is accomplished by the formation of a narcotic layer, the fluorescence intensity should be higher during the resting hours of the plant than during the working hours. To date there have not been sufficient experiments for a rigid test of this idea, but the few experiments made so far speak in favor of the hypothesis presented.

After several hours of irradiation in air, a leaf attached to the plant so that the food produced may flow off shows a fluorescence intensity 30 per cent smaller than that of a comparably treated excised leaf. A leaf kept in the dark for several days with its stem in water has a relatively weak steady state fluorescence, whereas a leaf treated in the same way but fed with sucrose for half an hour before irradiation (by putting its stem in a concentrated sucrose solution) shows a stronger fluorescence. This agrees with the observations of Korsanoff and others that the presence of an ample food supply in the plants accentuates the degree of temporary inhibition.

In a recent study of the light-induced limitations of photosynthesis, Stålfeld presented indications that large reductions in rate occur only if CO_2 limitation is responsible for saturation (this is the normal condition of higher plants exposed to sunlight in air). The author also gives experimental reasons for the assumption that the effect may be caused or accompanied by changes in acidity in the plant. These results, which have to be corroborated, are by no means in conflict with the hypothesis presented in the first part of this section. The rise in the acidity would favor the adsorption of an acidic narcotic on the chlorophyll-protein surface. The reduction in the concentration of CO_2 complexes caused by CO_2 limitation would have the same effect because of the decrease in competition of the complexes for places on the chlorophyll.

The depression of the rates might also develop in the following way: During hours of high photosynthetic activity the concentration of easily-oxidizable substances will rise, and accordingly they will compete increasingly with the O_2 -liberating enzyme for the photoperoxides. Thereby the production of the narcotic will increase and the photosynthetic rate will be decreased. The time course of the depression, however, indicates that probably increasing deactivation of the O_2 -liberating enzyme accompanies rising food supply. This may be achieved either by a rising permeability of the cell walls to the inactivating poison or by a higher production rate of the poison induced by the accumulation of food. More experiments, in which the time course of fluorescence and of the photosynthetic rate are studied simultaneously, are necessary to prove or disprove the idea that the formation of a natural narcotic is the factor which regulates the amount of photosynthetic activity.

RELATION BETWEEN PHOTOREDUCTION AND FLUORESCENCE OF
CHLOROPHYLL IN THIORHODACEAE (PURPLE BACTERIA)

The relation between the fluorescence of bacteriochlorophyll and photosynthetic activity has been extensively studied by Wasink, Katz, and Dorrestein. These authors found that, in contrast to the behavior of the fluorescence in green plants, the curve of fluorescence yield rises steeply at light saturation. Their explanation is based on the assumption that any rise in fluorescence yield is caused by a deficiency of those substances which, through contact with the chlorophyll, use the excitation energy for photochemical purposes. From the absence of a strong rise of the fluorescence yield at light saturation in green plants they concluded that an unknown substance is present in great abundance which, after activation by the light, is able to reduce CO_2 . They also see in the presence of a strong rise of the fluorescence yield at light saturation in purple bacteria a proof that the comparable substance in these cells is not available in abundance. On the other hand, the present author is convinced that a strong rise of the fluorescence yield, whether in green plants or purple bacteria, is always caused by the formation of a natural narcotic. The following pages present the reasons for his conviction.

STEADY STATE OF THE RATES OF PHOTOREDUCTION AND OF FLUORESCENCE
IN PURPLE BACTERIA

According to the interpretation now generally accepted (see van Niel, Kluyver, Franck, Gaffron, and Rieke), the principal difference between photosynthesis and photoreduction lies in the fate of the photoperoxides. In photosynthesis an enzyme decomposes them and thus liberates oxygen, while in photoreduction they react with hydrogen or hydrogen donors to form water. The main experimental support for this interpretation is given by measurements of the quantum yield. Although the energy needed for the over-all reaction in photoreduction (formation of carbohydrate from CO_2 and water in the presence of hydrogen) is practically zero, Rieke proved rigorously that in the alga *Scenedesmus* the transition from photosynthesis to photoreduction takes place without any change in the quantum yield. The photochemical steps of these two processes are, therefore, identical. The major difference between the process in purple bacteria and that in green plants is that the enzyme in green plants, which liberates oxygen from photoperoxides, is replaced in the purple bacteria by a hydrogenase which reduces the photoperoxides with hydrogen donors and forms water. It is, therefore, reasonable that the three enzymatic reactions which influence saturation in green plants do the same in purple

bacteria. The first adds CO_2 to another molecule before the photochemical steps take place; the second stabilizes the primary photochemical products; and the third reduces the photoperoxides by oxidation of hydrogen donors.

The enzymatic formation of CO_2 complexes can not be the limiting factor for saturation because saturation is connected with a strong rise in the fluorescence yield. It may be recalled that in green plants a removal of CO_2 complexes from the chlorophyll causes a rise in the fluorescence yield only if a narcotic is made by photooxidation. Since purple bacteria are observed only in O_2 -free atmospheres, photooxidation must be absent and the rise can not be caused by denudation of the chlorophyll.

A comparison between saturation curves and the curves of fluorescence intensity in purple bacteria shows that in practically all cases the fluorescence yield rises strongly at light intensities above the saturation intensity. If the enzymatic reaction by which the photochemical products are stabilized were responsible for saturation, there would not be an increase in the fluorescence yield at saturation, since no change of the concentration of photosensitive substances in contact with the chlorophyll would result at saturation. Therefore, the second enzyme is not responsible for saturation.

However, the strong rise of the fluorescence yield observed at saturation can be explained by the limitations imposed by the third enzymatic reaction, the one which transfers oxygen from the photoperoxides to hydrogen donors. This reaction can become limiting if not enough hydrogen donor is present, or if, in spite of a surplus of donors, the enzyme involved in the reaction limits the rate of removal of the photoperoxides. In green plants a strong fluorescence rise occurs during the induction period when the peroxides are not quickly removed by enzymatic action. The same should occur in purple bacteria, with the difference that, in this case, the failure to efficiently remove the photoperoxides is not a transient phenomenon but a permanent condition of the photosynthetic process at light saturation. The processes which occur when the removal of photoperoxides is the limiting factor responsible for saturation will be carefully analyzed in the following paragraphs.

In this particular case, we must differentiate between the observed saturation of the over-all gas exchange and the true saturation of the partial processes of reduction of CO_2 complexes and production of photoperoxides. The rate and light intensity values at saturation will be much larger for the last mentioned processes than the corresponding values for the over-all reaction. The surplus production of reduced substances and photoperoxides will not influence the net result of the over-all reaction because it is wiped

out by back reactions of reduced and oxidized substances with each other.² However, it is by no means necessary that these back reactions be confined to the freshly formed products of photosynthesis. Reaction between the photoperoxides and other oxidizable substances will not change the photosynthetic quotient so long as these other substances have the oxidation level of carbohydrates. Even if a small portion of the oxidation processes carried out by the photoperoxides involves substances whose oxidation state differs from a carbohydrate, the photosynthetic quotient will not deviate from the theoretical more than is experimentally observed. Therefore, although the narcotic must be an intermediate oxidation product which loses its narcotic properties as the result of further oxidation by photoperoxides, it is not necessary to assume that the oxidation reaction by which it is made is a reaction between carbohydrates and photoperoxides.

Let us now consider the conditions governing production of this narcotic. It is assumed that there are three major reactions which remove peroxides; the enzymatic reactions with hydrogen donors, the formation of narcotics, and the destruction of narcotics. The latter two occur only when the production of photoperoxides exceeds the capacity of the first. If the reaction between hydrogen donors and photoperoxides is responsible for saturation, the narcotics will start to be formed from the excess photoperoxides as soon as the light intensity approaches saturation. Its concentration will increase from there on with rising light intensities until at high intensities, which may surpass considerably the ones necessary for saturation, an equilibrium concentration of the narcotic is reached where the rates of its formation and its destruction caused by the same agent become equal. Thereafter the concentration of the narcotic will remain constant, and it will be independent of further changes in photoperoxide production caused by increases in the light intensity. Consequently, the fluorescence curve will rise linearly in this region. Furthermore, changes in the reaction rates between the hydrogen donor and photoperoxides imposed by changes of external conditions will have practically no influence on the slope of this linear part of the fluorescence curve, provided that the hydrogen donor reaction is the only cause for saturation.

We expect then that if the hydrogen donor reaction is varied, a set of curves will be observed in which the fluorescence curves

² This way of elimination of photoperoxides is by no means a hypothesis made only to explain the fluorescence phenomena in purple bacteria. It is, rather, an old assumption used by Gaffron for quite other reasons in his general scheme of photoreduction.

will be linear and parallel to each other at high light intensities. The proviso mentioned, i.e., that the reaction between hydrogen donors and photoperoxides shall be the only cause of saturation, is identical with the postulate that the percentage of peroxides removed by hydrogen donors is small at high light intensities. In that case, even great changes in the rate of the reaction will have very little influence on the amount of peroxides available for production and destruction of the narcotic. If, on the other hand, the maximum rate of the reaction between hydrogen donors and photoperoxides is comparable to the maximum rate of CO_2 formation or to the maximum rate of the enzymatic stabilization of photochemical primary products then, even at high light intensities, the final slope of the fluorescence intensity curve will not be as steep as in the previous case. Finally, if the maximum rate of the reaction between hydrogen donors and peroxides is greater than that of the other enzymatic reactions, there will be no rise of the fluorescence at exciting light intensities above saturation. It will have the same low value at all intensities which it has at very low intensities.

We will now compare the results of these theoretical considerations with the experimental results of Wassink, Katz, and Dorrestein (3). These authors observed photosynthetic saturation curves of the normal type with different kinds of hydrogen donors. If the concentration of any of the hydrogen donors used falls below a certain value, the saturation rate will go down, and accordingly is reached at lower light intensities. The maximum saturation rate is about the same for the donor hydrogen gas and for thiosulfate if the pH of the suspension is kept at 6.3, but at the pH of 7.6 the saturation rate is higher for molecular hydrogen, while the rate with thiosulfate becomes smaller. The reason for the influence of the pH on the saturation rates is not known, but it may be surmised that the effect is caused by changes in the permeability of the outer membranes of the cell for the hydrogen donors.

Figure 16.20 shows the influence on the fluorescence curves of the concentration of the reducing substrate, thiosulfate. The lower the thiosulfate concentration, the lower the light intensity at which the rise in the fluorescence intensity starts. At thiosulfate concentrations up to one per cent the curves are linear at high light intensities and the final slopes are all equal. In accordance with theoretical expectations, these facts indicate that even at high concentrations 0.5–1 per cent of thiosulfate the reaction between the hydrogen donors and the photoperoxides is alone responsible for saturation. The same is true when hydrogen gas at very low concentrations is used as reducing substrate (Fig. 16.21). However,

when a high concentration of hydrogen gas (15 per cent) is used, it is no longer permissible to conclude that the reaction between hydrogen donors and photoperoxides is alone responsible for saturation. One or both of the other enzymatic reactions begin to contribute to the limitations. Consequently the rise in fluorescence intensity starts at higher incident light intensities and, even at the highest light intensities, never reaches the steepness of slope observed in the preceding cases.

Similar observations have been made on the dependence of the rate curves and fluorescence curves upon temperature. Changing the temperature will, of course, influence all dark reactions. If the temperature is lowered, the saturation rates and saturation intensities will also become lower. If the difference between the temperature coefficients for the different dark reactions is not too great, the set of curves measured at different temperatures and the same reducing substrate concentration should be similar to the set measured at one temperature and different concentrations of the substrate. With thiosulfate this relationship is observed. However, with hydrogen gas as reducing substrate the curves look somewhat different, especially at higher temperatures. As discussed above, in the presence of hydrogen, saturation is caused not only by the limitations of the enzymatic reaction between hydrogen donors and photoperoxides, but also by limitations of the other enzymatic reactions. The variation of the measured curves with temperature indicates that in this case the reaction between hydrogen donors and photoperoxides has a larger temperature coefficient than the other dark reactions, thus further complicating the situation.

Next we have to discuss the influence of the CO_2 concentration on the rate curves and on the fluorescence curves under conditions where a surplus of hydrogen donors is present. Figure 16.22 presents rate curves measured at different CO_2 concentrations. The shape of the curves and the dependence of saturation rates on CO_2 concentration is the usual one. Of importance is the fact that, even if no CO_2 is added to the bacterial suspension, an uptake of hydrogen occurs which is by no means negligible. This hydrogen uptake is about 15 per cent of the hydrogen uptake at saturation in the particular case reproduced in the curve. From other data one can deduce that the uptake of hydrogen in the absence of added CO_2 is usually about 10 per cent of the saturation uptake. This shows that CO_2 made by fermentation is present in a concentration which cannot be neglected, and that the condition which Wassink and co-workers call, "no carbon dioxide," actually represents about 10 per cent of the limiting concentration of CO_2 .

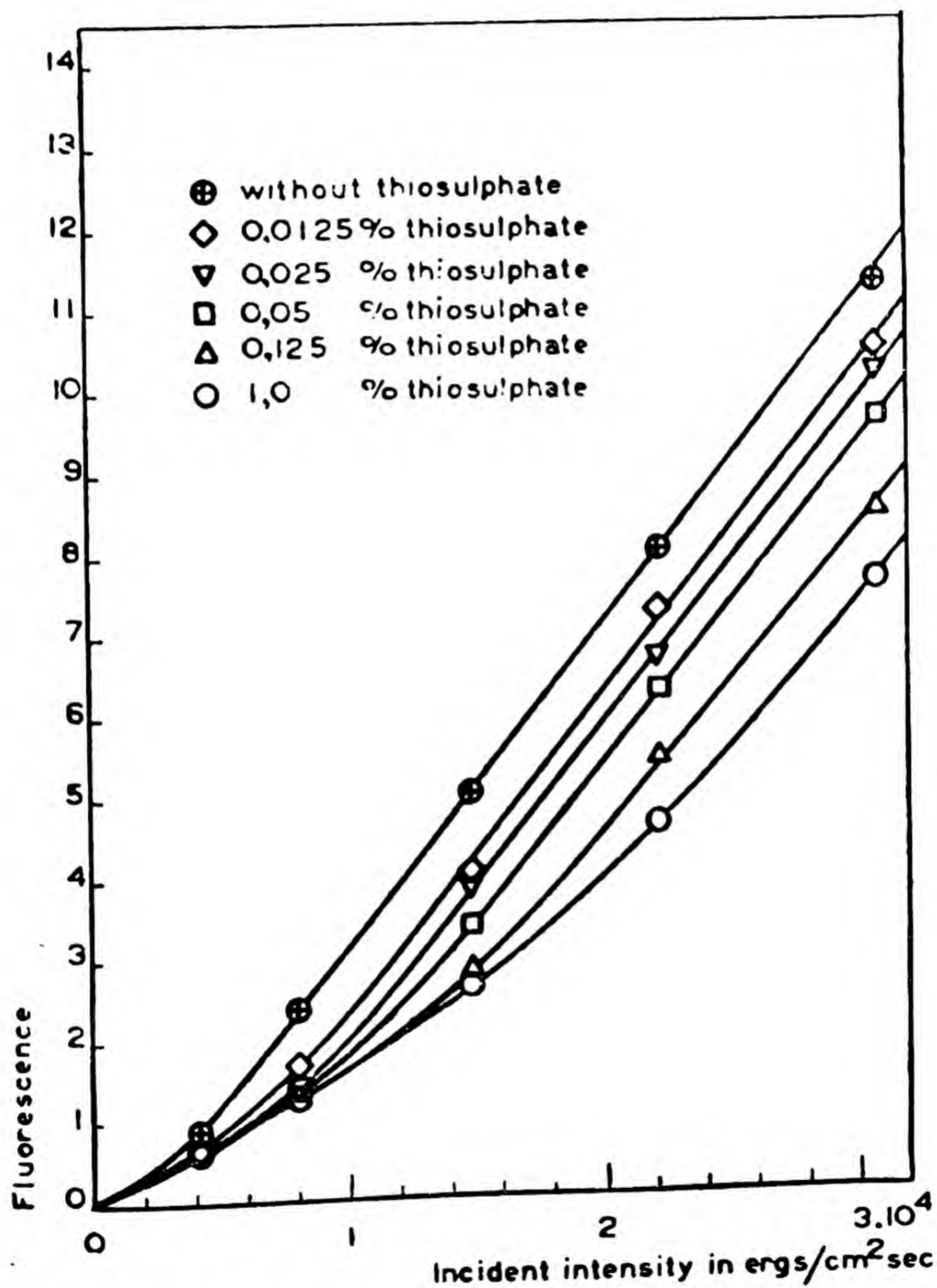


FIG. 16.20—Influence of the concentration of thiosulphate upon the intensity of fluorescence (CO₂—5 per cent, pH 6.3, 29°C.)

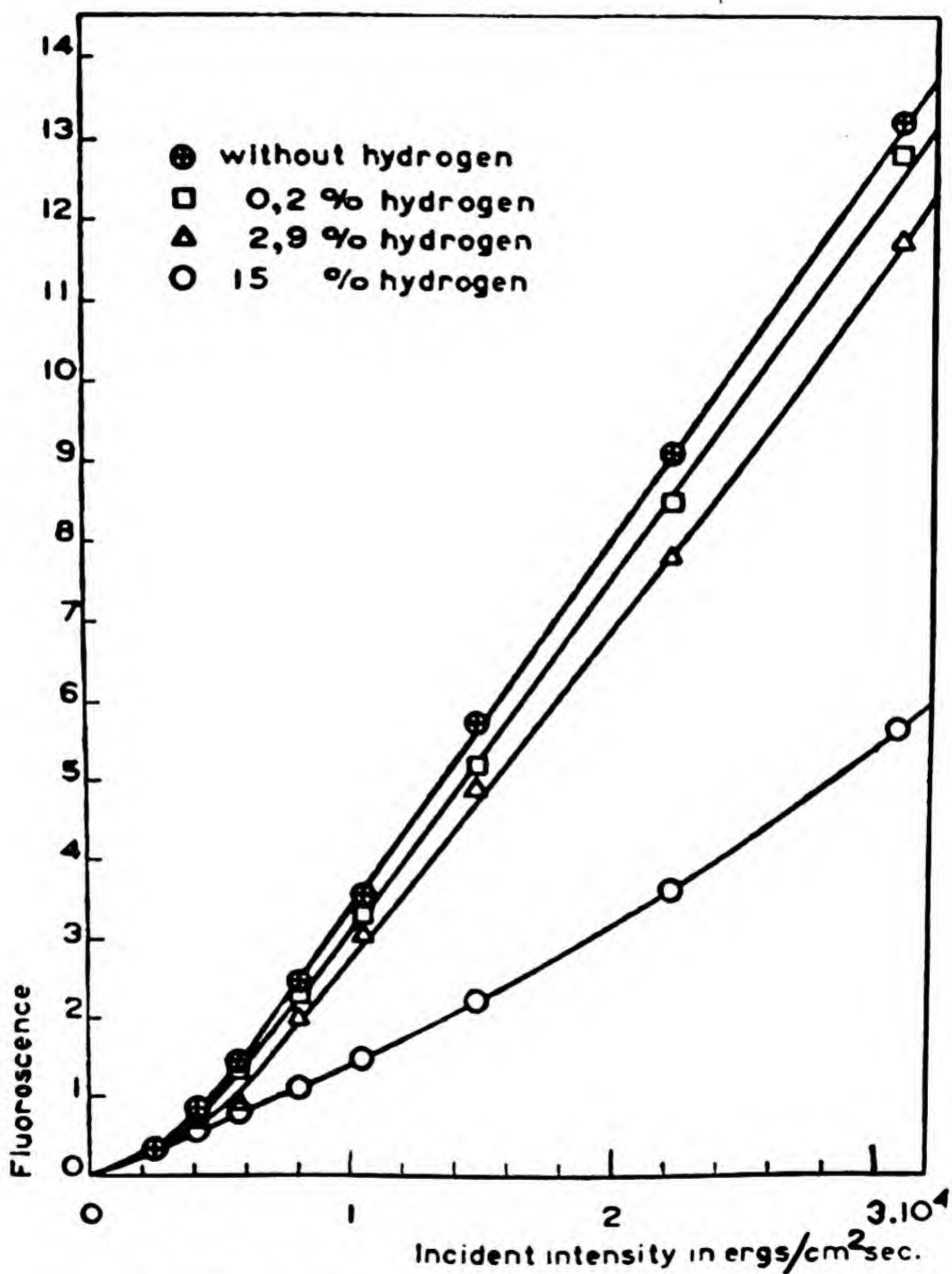


FIG. 16.21—Influence of the concentration of hydrogen upon the intensity of fluorescence (CO₂—5 per cent, pH 7.6, 29°C.)

An example of the influence of CO_2 concentrations on fluorescence curves measured with hydrogen gas as reducing substrate is presented in Figure 16.23. When the concentration of CO_2 is high, the curve of fluorescence intensity rises with the intensity of the exciting light and deviates widely from linearity, while without extra CO_2 , the deviation is much less. The latter curve shows slightly higher fluorescence intensity at low intensities of the exciting light than does the one with added CO_2 so that the curves cross

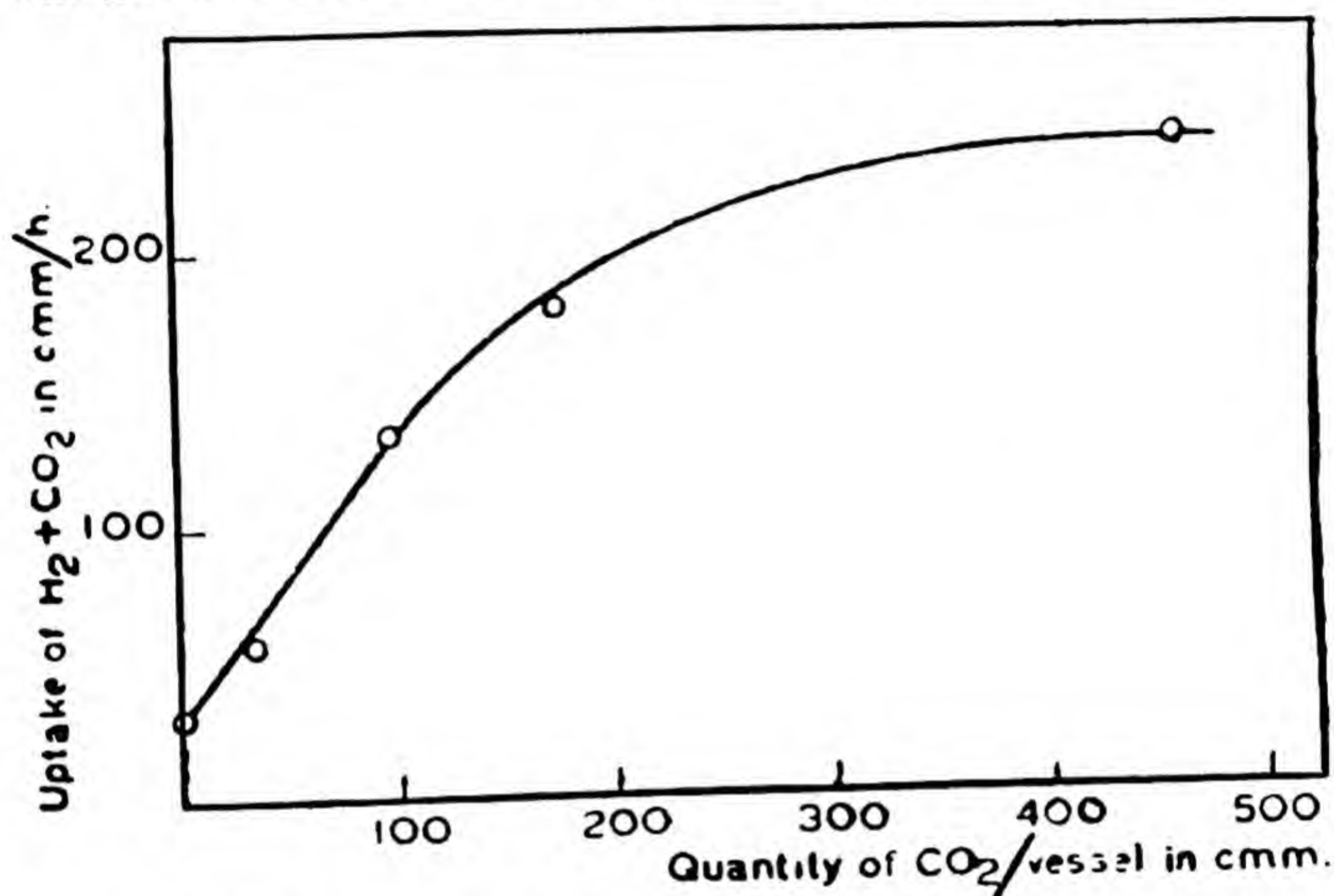


FIG. 16.22—Influence of CO_2 upon the rate of photosynthesis (H_2 —15 per cent, pH 6.3, 29°C ., high light intensity).

each other. This fact and the presence of some curvature in the curve measured without added CO_2 indicates that even with such strong CO_2 limitation a small amount of narcotic is adsorbed on the chlorophyll-protein surface. The amount of narcotic present may, however, be very small since with such a low concentration of CO_2 and rising light intensities will remove practically all of the CO_2 complexes and leave the entire surface of the chlorophyll to the narcotic. While by no means impossible, it would be astonishing if, in spite of the exceedingly strong competition of the hydrogen donor for the peroxides, the narcotic present under these conditions was an oxidation product made by the reaction of photoperoxides. Another possibility to explain its presence is the assumption that it is made by fermentation. Evidence showing that such a narcotic is

produced in purple bacteria will be presented in the following section.

Further information may be obtained from observations on the influence of cyanide. Even if added in relatively small concentration, it reduces the saturation rates. If the enzyme involved in the formation of CO_2 complexes in purple bacteria is as sensitive to cyanide as it is in green plants, its poisoning could be the reason for the strong influence on the saturation rate. If only this enzyme is sensitive, the fluorescence curve should be straighter in the presence of cyanide and the fluorescence intensity should be lower (Fig. 16.23). On the other hand, it is unknown how sensitive the enzymatic reaction between hydrogen donors and photoperoxides may be to cyanide. If this enzyme is the more sensitive one, then the curve measured in the presence of cyanide should be more bent and the fluorescence intensity should be greater. Actually the observations with low cyanide concentrations give no clear-cut answer. The influence on the shape of the fluorescence curve is much less than that on the saturation rates. This would seem to indicate that both enzymes may have about the same sensitivity toward cyanide (Fig. 16.24). Of greater significance, however, is the influence of cyanide when used in a concentration so high that photosynthetic activity is almost completely inhibited. In this case the fluorescence curve is almost a straight line, with a very low fluorescence yield. According to our hypothesis, such behavior is to be expected because, if no or almost no photoperoxides are made, no narcotic can be produced.

Finally, we have to discuss the results which Wassink and co-workers regard as definite proof in favor of their assumption that CO_2 is not reduced in contact with the chlorophyll but rather by an independent reducing substance which is made photochemically. The observations are studies of the influence of the CO_2 concentration on the fluorescence curves when no hydrogen donor is present (Fig. 16.25). The presence or absence of added CO_2 has no influence on the fluorescence curve in the absence of hydrogen donor. One might interpret this, as do Wassink and Katz, as an indication that the presence in high concentration or total absence of CO_2 cause no change in the fluorescence curve measured in the absence of hydrogen donor. Such an interpretation would indeed favor their hypothesis.

However, as previously discussed, CO_2 is present even if not added externally (Fig. 16.22). Therefore, the experiment shows only that the fluorescence intensity is quite independent of the CO_2 concentration down to low values. This is exactly what would

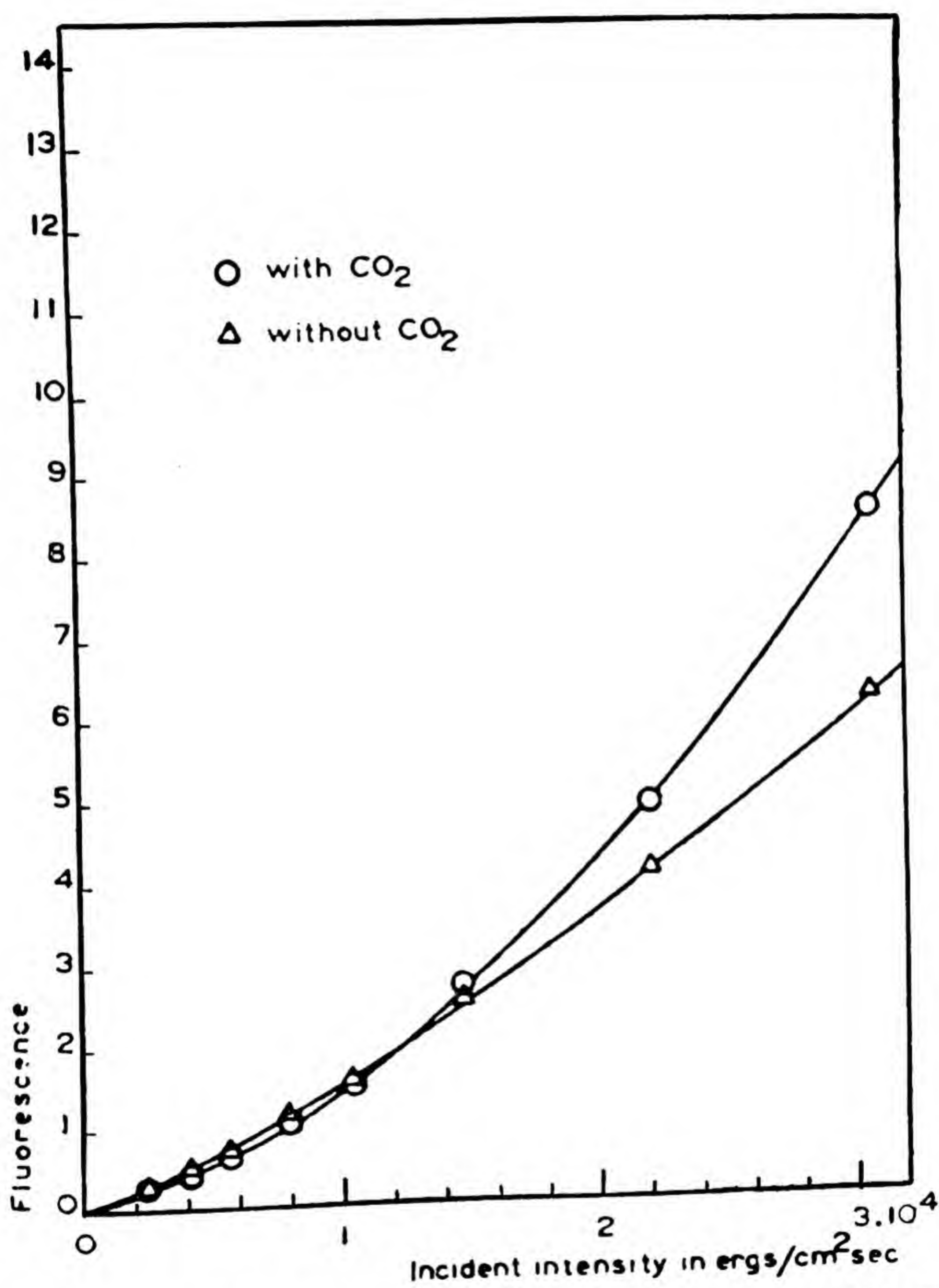


FIG. 16.23—Influence of CO₂ upon the intensity of fluorescence (H₂—15 per cent, pH 6.3, 29° C.).

be expected according to our theory when hydrogen donors are absent. In such a case the enzymatic reaction between hydrogen donors and photoperoxides is the limiting reaction at low as well as at high CO_2 concentrations. Consequently saturation occurs at the same low light intensity no matter whether the surplus of CO_2 is great or small, narcotic is formed when the saturation intensity is reached, and the concentration of the narcotic is independent of the production rate of photoperoxides present. Therefore, no change in the shape of the fluorescence curve is expected to occur from change of the CO_2 concentration. Of course, to insure the above, sufficient CO_2 must be present to provide a surplus of photoperoxides over the amount which can be taken care of by the internal hydrogen donors. However, a very low concentration of CO_2 will fulfill this condition.

Addition of cyanide will also be expected to have little or no influence on the shape of the fluorescence curve in the absence of added CO_2 and hydrogen donor. This is, indeed, the case for smaller cyanide concentrations (cf. Fig. 16.26). If, however, cyanide is added in a concentration sufficient to stop all photosynthetic activity, the shape of the fluorescence curve is changed. It approaches linearity and the fluorescence yield remains low. This too is in accord with our theory for if no peroxides are produced no narcotic can be made and, therefore, no rise of the fluorescence yield will occur. It may be mentioned that Wassink *et al.*, explain this result, unexpected from the viewpoint of their theory, by the hypothesis made *ad hoc* that cyanide of sufficiently high concentration acts as an acceptor of the excitation energy of the chlorophyll.

We refrain from discussing further results presented by these authors; for instance, the influence of urethane and sodium azide on photosynthetic rates and fluorescence. These data offer no particular difficulty in interpretation and would not broaden the discussion. Furthermore, as the results are not always consistent, more experiments should be done before a detailed discussion is warranted.

TRANSIENT PHENOMENA OF FLUORESCENCE INTENSITY IN PURPLE BACTERIA

Wassink and co-workers call preliminary some observations which they present concerning changes in the fluorescence intensity at the beginning of an irradiation period. Indeed, it is desirable to extend these observations by measurements under a greater variety of external conditions than have been used. However, it is important to see whether the phenomena observed so far fit the interpretation presented for the steady state of fluorescence in purple bacteria. The following discussion will show that while the observations are

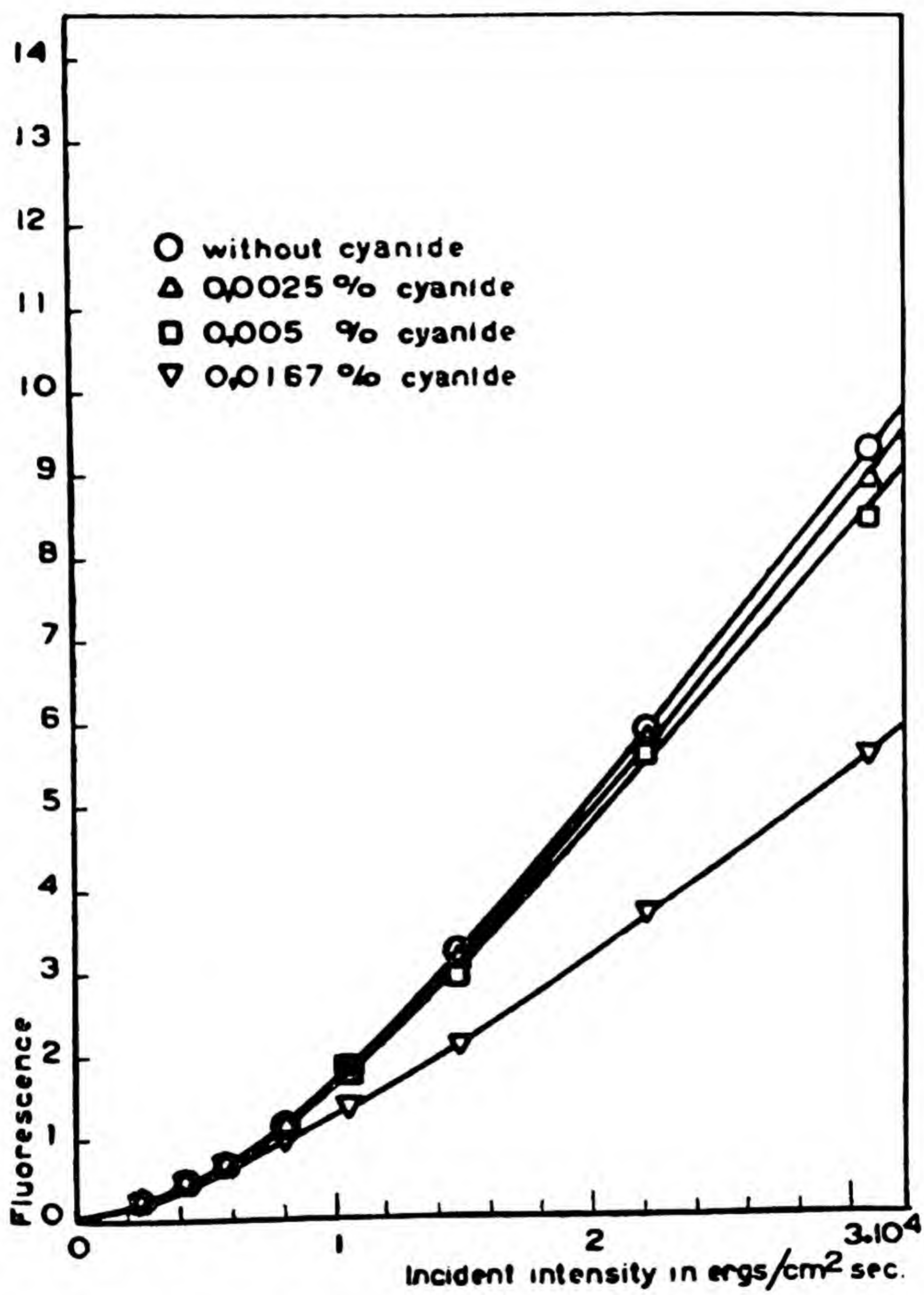


FIG. 16.24—Influence of cyanide upon the intensity of fluorescence (CO_2 —5 per cent, thiosulfate 1 per cent, pH 7.6, 29° C.).

in full accord with the picture we have offered, they do not support the hypothesis of Wassink and co-workers. These authors have to introduce special assumptions for the interpretation of some of the observations while others remain unexplained.

Figure 16.27 shows the general type of fluorescence-time curve obtained from observations made at normal temperature in the presence of a hydrogen donor and at a high intensity of irradiation. The curve has a great similarity to the comparable one observed with green algae under anaerobic conditions. The explanation used in the case of the algae can be applied unchanged to the interpretation of the fluorescence curve in purple bacteria. In both cases, the enzymatic removal of the photoperoxide is the limiting factor, and in both, two natural narcotics influence the fluorescence intensity. One narcotic is made by a reaction of photoperoxide with easily oxidizable substances, and the other is made as a product of anaerobic fermentation. The latter one is responsible for the unusually high fluorescence yield at the starting point. (Note that the intensity at the starting point in Figure 16.6 lies much higher than the intensity after the steady state of fluorescence is reached.) Its gradual removal by oxidation with the help of the photoperoxides causes the fluorescence intensity to decline evenly with time until a low equilibrium concentration of the narcotic and a correspondingly low fluorescence intensity is reached. The other narcotic, made by the reaction of peroxides with oxidizable substances, quickly enhances the fluorescence intensity because its concentration rises from zero in the dark to the value present at equilibrium in the light. The observed curve is then the resultant of the effects of both narcotics on the fluorescence intensity. Whether the fluorescence curve will start with a steep rise of the intensity or whether the curve will start its course with a declining slope depends upon the relative rates of the formation of the one narcotic and of the destruction of the other by reaction with the photoperoxide.

The case represented by Figure 16.6 shows a rise at the start, indicating that the production of the one narcotic surpassed the destruction of the other. If a very high concentration of the fermentation-made narcotic were present at the start, it would compete with the hydrogen donors for the small quantity of photoperoxides, and its destruction would begin immediately upon the start of illumination. The photosynthetic activity would be very small at the start. Therefore, during the first moments of irradiation the reaction between photoperoxides and hydrogen donors would not be limiting. However, the photosynthetic activity would increase autocatalytically with the removal of the fermentation narcotic, and

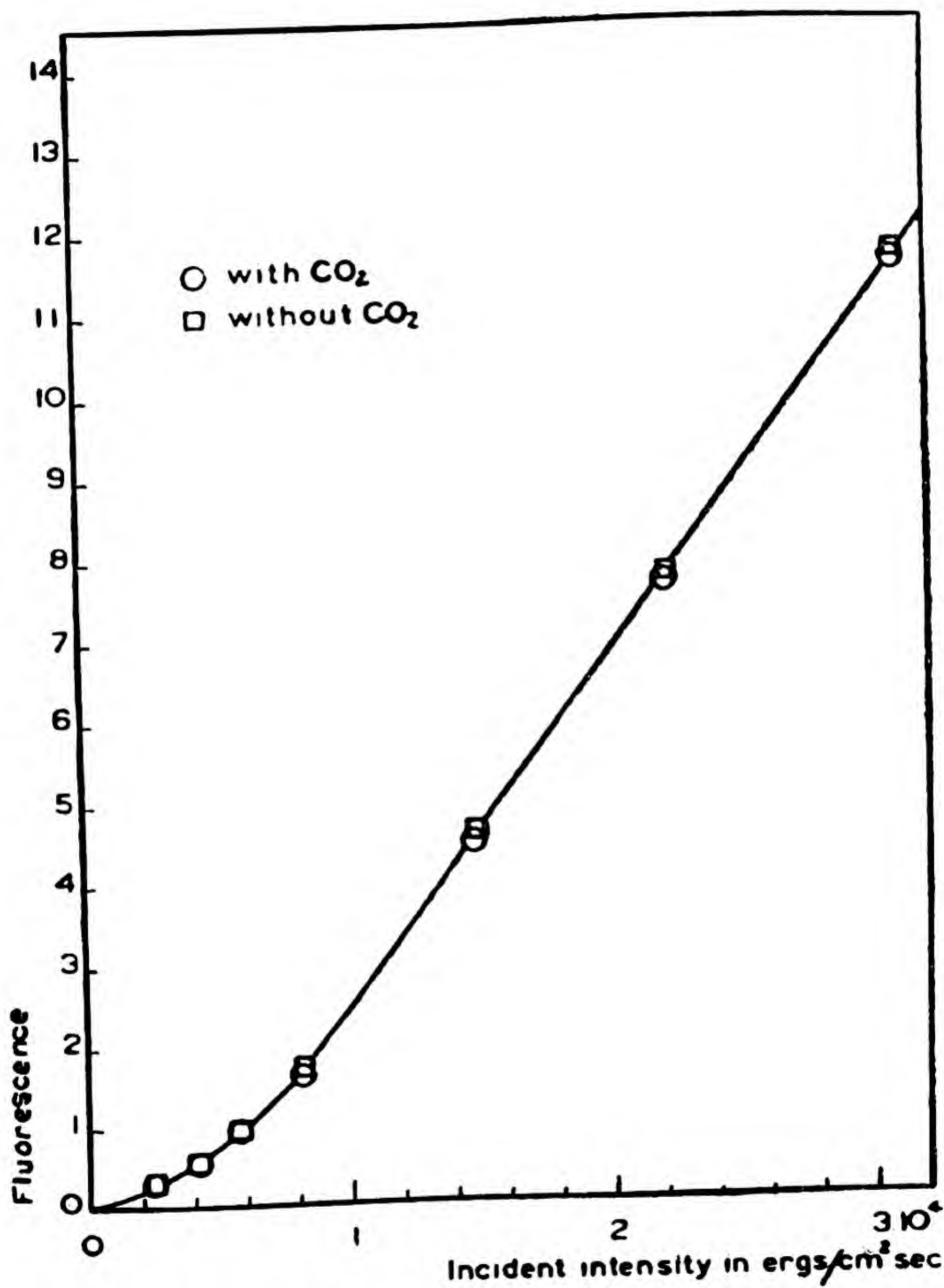


FIG. 16.25—Influence of CO₂ upon the intensity of fluorescence in the absence of a hydrogen donor (pH 6.3, 29° C.).

the reaction between hydrogen donors and photoperoxides would soon become limiting. This would result in the production of the narcotic formed by oxidations involving the photoperoxides. Accordingly, a temporary decline at the beginning of irradiation may be quickly followed by a temporary rise of the fluorescence curve. In other cases the decline caused by the removal of the fermentation narcotic may surpass at every moment the rise which the production of the second narcotic would bring about. Then the curve of fluorescence intensity would fall monotonously.

Wassink, Katz, and Dorrestein observed these three types of curves, some with a rise at the beginning, some with a delayed rise, and some with a monotonous decline. Our interpretation not only explains how such anomalies can occur at the beginning of the irradiation period but also the conditions under which the one or the other of these types will be observed. So far as can be seen from the data presented in the Dutch authors' paper, there is an agreement between our theoretical prediction and their observations. They state specifically that, for the time being, they see no way to explain the phenomena. They also find it difficult to understand the general decay of the fluorescence intensity during the first minutes of irradiation. The similar time course of fluorescence observed with algae in an atmosphere of nitrogen was explained by them on the assumption that the fluorescence of the chlorophyll is quenched directly or indirectly by oxygen made by the process of photosynthesis. Since photoreduction in purple bacteria proceeds without oxygen production, the fall of the fluorescence intensity must be explained in another way. The authors comment only that the changes of the fluorescence intensity "bear the character of shifts in the state of oxydo-reduction of the transfer surface." Why the fluorescence decreases in *Chlorella* by a shift to the oxidized side and in purple bacteria by a shift to the reduced side is left by them to later "more profound" studies of these phenomena.

It seems unnecessary to discuss the slowing down of the decay of the fluorescence caused by lowering of the temperature or by the addition of ethyl urethane. It is evident that, according to our theory, any factor which diminishes the rate of peroxide production in the light will slow down the rate of removal of the fermentation narcotic. However, we have to discuss the interesting fluorescence-time curves measured in the absence of external hydrogen donors by these authors.

Figure 16.28 gives a set of curves of fluorescence in the absence of hydrogen donor measured in succession, with dark periods of varied duration separating one measurement from the next. The

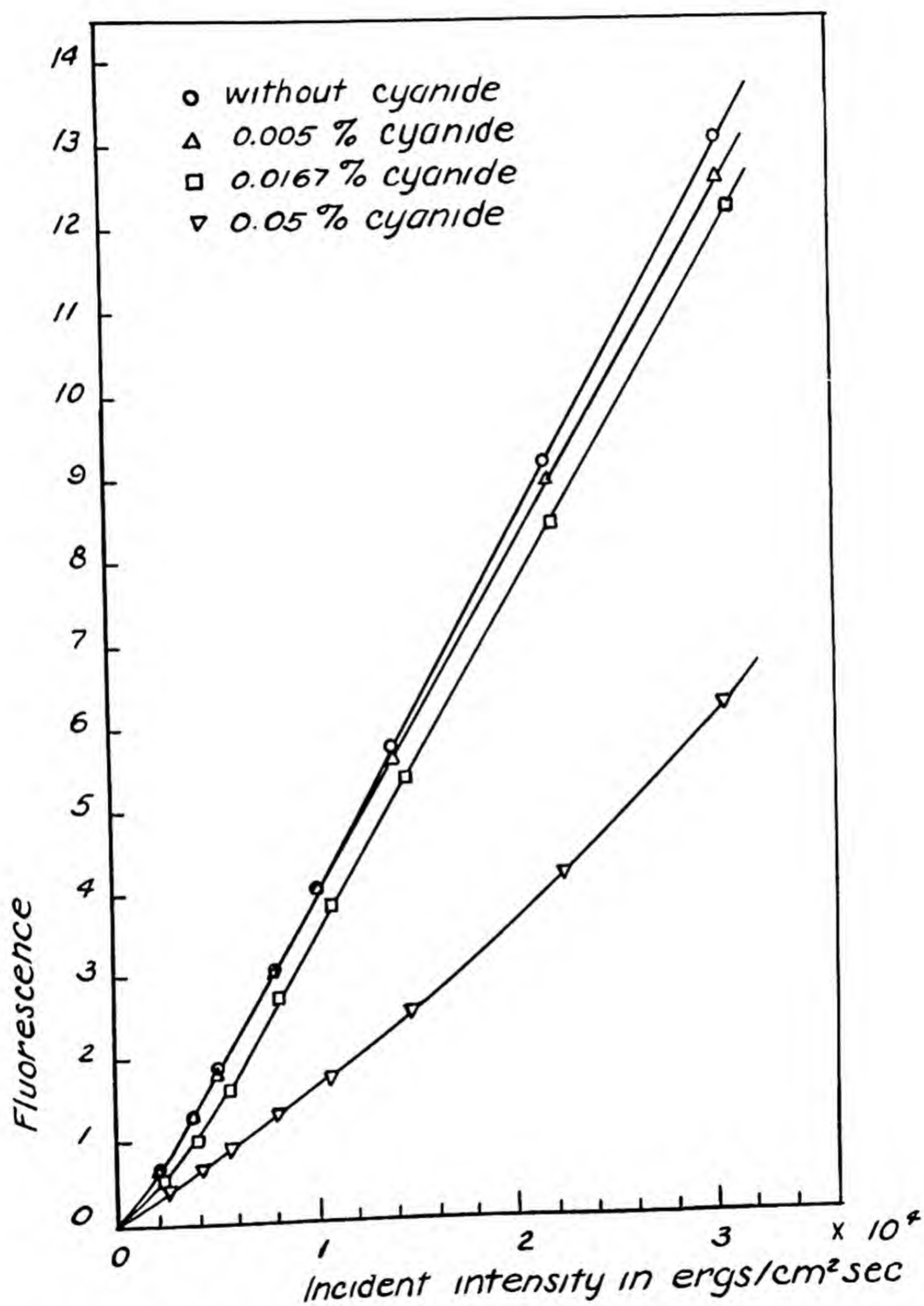


FIG. 16.26—Influence of cyanide upon the intensity of fluorescence in the absence of a hydrogen donor (CO_2 —5 per cent, pH 6.3, 29°C.).

fifth curve, measured after a quite short dark pause, has the simplest shape. The fluorescence intensity rises from the start for about a minute and remains constant from there on. This type of curve is expected to occur if the narcotic made by the reaction between photoperoxides and some oxidizable substance has a strong influence on the time course of fluorescence while the narcotic made by fermentation is practically absent. Indeed, both conditions are fulfilled.

The concentration of the fermentation narcotic must be negligible after a dark period of only two minutes following quite strong irradiation, and the rate of formation of the narcotic produced by the photoperoxides must be high because of the absence of external

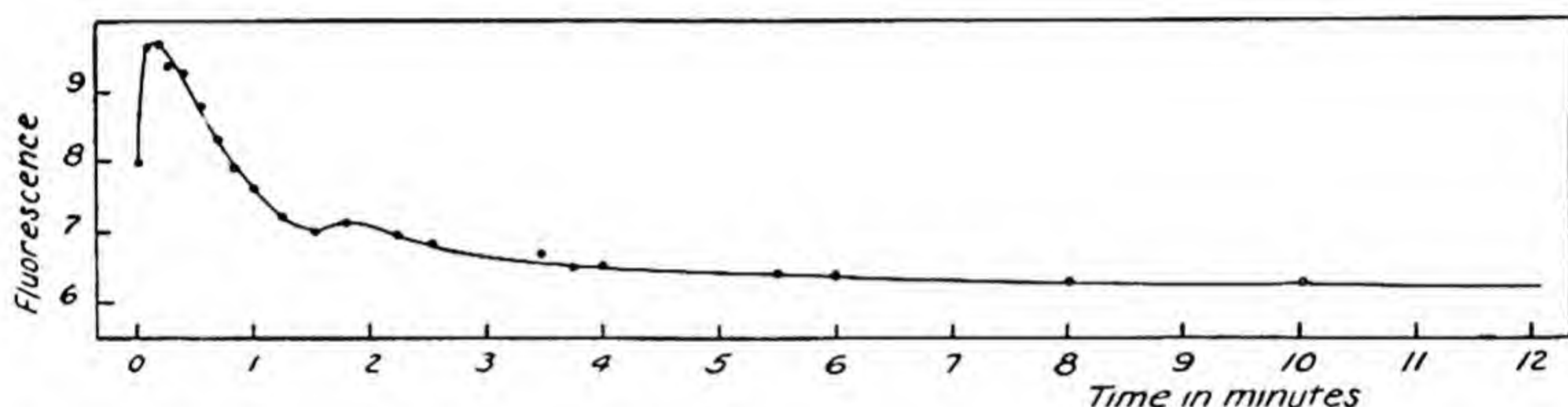


FIG. 16.27—Initial changes of fluorescence after the start of an illumination. General type of curve. $I = 3.10 \times 10^4$ ergs/cm.²/sec. (CO₂—5 per cent, thiosulfate 1 per cent, pH 6.3, 29° C.).

hydrogen donors. Internal hydrogen donors, if present at all, should be in very low concentration because their rate of metabolic production is, just as that of the fermentation narcotic, too small to give an appreciable accumulation in only two minutes of dark time. The situation is quite different for the curve taken during the first illumination period when a long dark period had preceded the experiment. Both metabolic products, the fermentation narcotic and the internal hydrogen donors, will have appreciable concentrations under these conditions. Consequently, the fluorescence curve starts like a normal one taken in presence of hydrogen donors and fermentation narcotic (cf. the corresponding parts of the curve of Fig. 16.1). However, the curve deviates from the normal one as soon as the supply of internal hydrogen donors is exhausted.

The fluorescence intensity shows a new rise because the rate of production of the oxidatively-formed narcotic will rise when the competition of the hydrogen donors for the photoperoxides stops. Wassink and co-workers realized, of course, that this rise is caused by lack of hydrogen donors and were able to show that similar rises in the fluorescence intensity can be produced in the

presence of hydrogen gas as donor, if, at a certain moment, the shaking of the vessel containing the bacterial suspension is stopped. Again lack of hydrogen donor is the reason because, without stirring, the hydrogen will be consumed much faster by the bacteria than it is replaced by diffusion.

SUMMARY

1. A survey is presented of the experimental evidence proving that in green plants and in purple bacteria a rise of the fluorescence yield of the chlorophyll is always connected with a limitation of the photosynthetic activity. For the explanation of this relation the

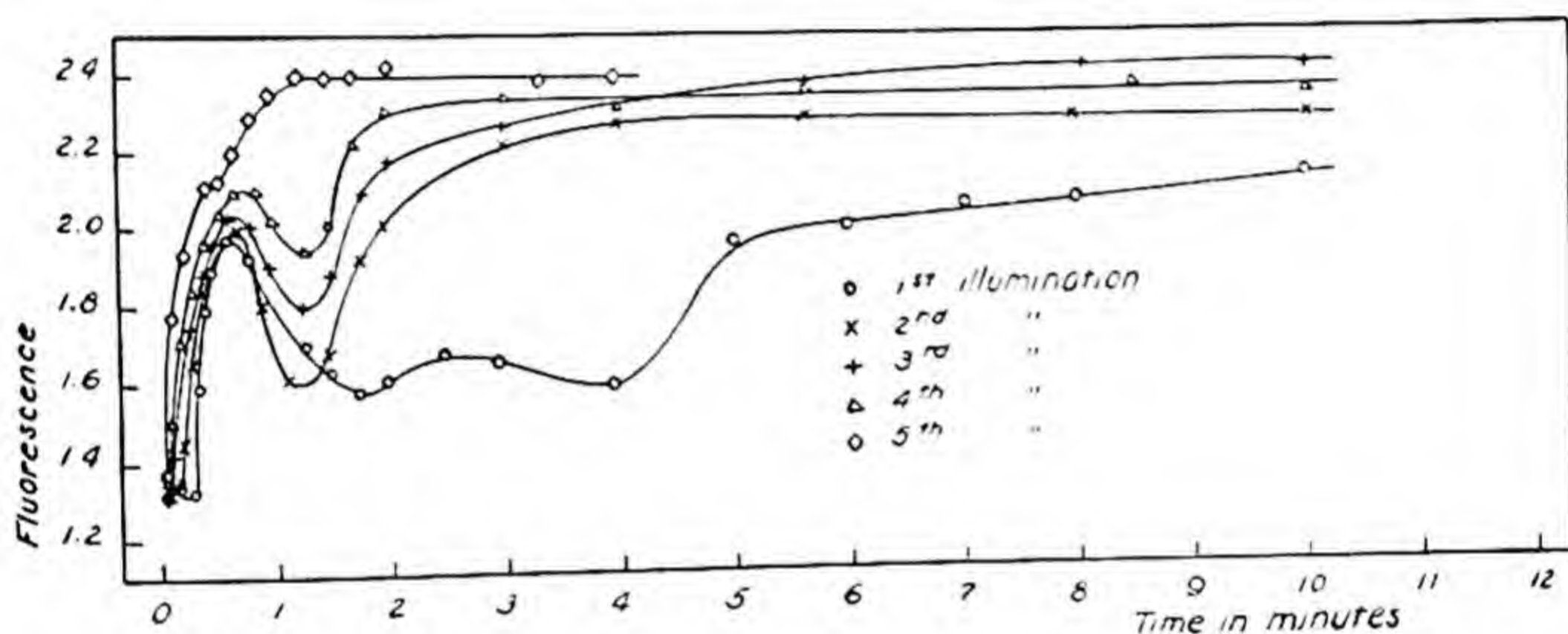


FIG. 16.28—Initial changes of fluorescence after the start of an illumination. $I = 1.0 \times 10^4$ ergs/cm.²/sec. Repeated illumination in the absence of a hydrogen donor with dark periods of 10, 10, 10, and 2 minutes. (CO_2 —5 per cent, pH 6.3, 29° C.)

hypothesis is advanced that narcotic-acting substances can be produced in plants and adsorbed at the chlorophyll-protein surfaces, thereby reducing the photochemical activity of the chlorophyll and enhancing its fluorescence yield.

2. The hypothesis postulates that the narcotics are intermediate oxidation products of easily oxidizable substances, both made and destroyed by oxidation processes. According to the prevailing conditions the oxidizing agents are: (a) molecular oxygen activated by light (photooxidation); (b) photoperoxides made as an intermediate product of photosynthesis or photoreduction.

One has to assume further that natural narcotics can also be produced under anaerobic conditions by the process of fermentation. The natural narcotics are apparently plant acids. They can be removed from the chlorophyll-protein surface by making the interior of the cells alkaline.

3. The phenomena of the short induction period in green algae are explained by the assumption that a poison of the oxygen-liberating enzyme is formed by dark metabolism, and that this allows photoperoxides to accumulate during the first moment of irradiation and thus to form a narcotic. (Supporting evidence for the assumption of the poison formation, based on results of experiments of quite different character, is presented.)

4. The phenomena of the long induction period in green algae and in purple bacteria are explained by the slow oxidative removal of the narcotic formed in excess by fermentation during the preceding dark period.

5. The relation between the steady state of photosynthetic rates and of fluorescence yield including the effect of poisons, changes in temperature, substrate concentration, etc., are clearly explainable for green plants and for purple bacteria as a consequence of the competition between substances to be photosynthesized and narcotic substances for places on the chlorophyll surface.

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17.

The Pattern of Photosynthesis in *Chlorella*

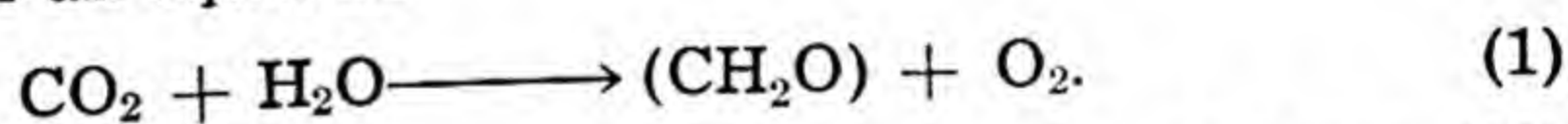
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THE GREEN ALGAE such as *Chlorella* have been used principally as an experimental material in the study of photosynthesis. The recent work of this laboratory has been directed toward a more careful scrutiny of other features of algal metabolism. The present paper will summarize some of the results, to be reported elsewhere in more complete form, and will consider in detail that particular part of the work which appears to have important bearing on the photosynthesis problem.

CONSIDERATIONS OF COMPARATIVE PHYSIOLOGY

The early work on photosynthesis in higher plants clearly established a quotient of -1.0 for the CO_2/O_2 gas exchange. This requires an over-all equation



Subsequent work on plant analysis and on the carbon balance (18) showed that the only rapidly accumulating materials of the CH_2O state of reduction are carbohydrates; hence the classical definition of photosynthesis as the "reduction of carbon dioxide to carbohydrate."

In 1919 Warburg (20) introduced simultaneously a new method of gas exchange measurement and a new experimental material, the green alga *Chlorella*. Values of the assimilatory quotient (CO_2/O_2) in many investigations of this organism cluster around the value -0.9 . This has been regarded only as a minor deviation from -1.0 , and it has always been presumed that the metabolism of the algae could be inferred from the more extensive data on higher plants.

Little attention has been paid to comparison of the physiology of

the algae with that of other photosynthetic forms. In cellular organization, in pigment systems, and in the use of water as a hydrogen donor the green algae are strikingly similar to higher plants. However, in their high nitrogen content, in the ability of some forms to adapt themselves to hydrogen donors other than water (9), and in the characteristic of producing new cells as the end product of photosynthetic metabolism, the green algae show closer physiological relationships to another group of organisms, the photosynthetic bacteria.

In at least two of the green algae the end products of photosynthetic metabolism are almost entirely new cells and exchangeable gases. This reproduction of photosynthetic machinery may also occur in young, growing leaves of a higher plant. In mature leaves, however, the photosynthetic machinery merely maintains itself, and its products are transported away. These are important considerations in the interpretation of data obtained on algae and higher plants.

Important ideas may be obtained also from consideration of the ecology and natural growth habitat of the algae. Such an approach has been used by Foster (7) to bring coherence to the interpretation of mold metabolism. Under the frugal nutritional conditions of their natural habitats the molds are highly efficient in converting substrates to cell material. When provided with abundant food supply, however, large amounts of substrate are converted via metabolic shunts to storage or excretory products. The green algae make up an ecologically versatile group. Many of them, such as those which inhabit soils, must be geared to a marginal economy. This certainly appears to be the case with *Chlorella*.

When provided with CO_2 and mineral salts *Chlorella pyrenoidosa* will grow at a light intensity of less than 10 fc. and reaches a maximum growth rate at less than 100 fc. (13). *Chlorella* for experimental use is often grown at low effective light intensities. Subsequent short-time experiments (1–2 hours) in the usual Knop's solution at photosynthesis-saturating light intensities usually yield an assimilatory quotient of -0.9 . During growth in long-time experiments, however, the assimilatory quotient is about -0.75 (Myers and Johnston, unpublished). These considerations lead to the expectation that the algae may have a variable metabolic pattern, and that under luxurious nutrient conditions (high light, CO_2 , etc.) metabolic shunt products may accumulate.

THE OVER-ALL METABOLISM OF CHLORELLA

The experimental methods, to be described in detail elsewhere, will be only summarized here. *Chlorella pyrenoidosa* (Emerson's

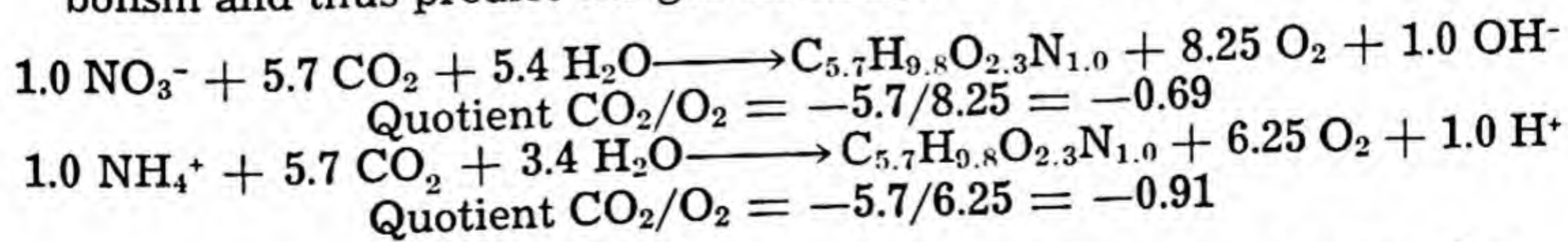
strain) was grown in a continuous culture apparatus (15) which yields uniform experimental material grown at a controlled light intensity. Most of the cells used in the present experiments were grown under an effective light intensity of about 40 fc. at a relative growth rate of 0.42 per day. This is a light-limited rate of growth (cf. 13).

Dark respiration of the harvested cells was studied by the direct method of Warburg, using the common respiration vessels with and without alkali. Photosynthesis was measured under 4 per cent CO_2 by the indirect method in duplicate rectangular flasks with different liquid:gas ratios. A temperature of 25°C . was maintained for both growth and manometric measurements. Light intensity for the measurements was provided by a bank of tungsten lamps operated from a voltage regulator and variable transformer. Low intensities were obtained by reduction of voltage since it had previously been established that the gas exchange rate and quotient at high intensity is the same in neon (red) light as in tungsten light. Nominal measurements of light intensity were made by a Weston photo-cell immersed in the bath. The light intensity varied several per cent between flask positions. In order to average out this variation in experiments at low light intensity, the positions of the flasks and manometers were reversed and pressure changes obtained for thirty-minute periods of constant rate before and after reversal.

It is necessary to summarize here a number of characteristics of over-all metabolism not yet available in the literature.

1. During photosynthetic growth in mass cultures, 95 per cent of the CO_2 and nitrate taken up can be recovered as cellular carbon and nitrogen. This is consistent with the carbon balance obtained by Krogh, Lange, and Smith (11) on *Scenedesmus*. Under the usual conditions of growth, therefore, excretory products are not appreciable and new cell material is the only metabolic product.

2. Elementary analysis on *Chlorella*, as grown in our experiments, yields 53.0 per cent C, 7.5 per cent H, 28.5 per cent O, 10.8 per cent N on an ash-free, dry-weight basis. On dividing by the appropriate atomic weights these percentages can be converted to the expression $\text{C}_{5.7}\text{H}_{9.8}\text{O}_{2.3}\text{N}_{1.0}$. If the nitrogen source is known, it becomes possible to write balanced equations for over-all metabolism and thus predict the gas exchange. For instance:



It is seen that the high nitrogen content leads to prediction of a

marked effect of the nitrogen source (NO_3^- or NH_4^+) on the gas exchange quotient.

3. An assimilatory quotient of -0.9 has often been reported for *Chlorella* in nitrate-containing media. Invariably this has been measured at high light intensity. If the gas exchange is studied at the same low intensity at which the cells have been grown, however, the average of many closely agreeing measurements gives remarkable confirmation of the predictions from cell analysis. These are summarized in Table 17.1. The low value of the quotient¹ on nitrate is confirmed in mass culture experiments at low light intensity.

TABLE 17.1
 CO_2/O_2 QUOTIENT FOR CELLS GROWN AT 40 FC. AND STUDIED AT 40 FC.

Nitrogen Source	CO_2/O_2 by Manometric Measurement	CO_2/O_2 Calculated from Cell Analysis
NO_3^-	-0.68	-0.69
NH_4^+	-0.94	-0.91
$\text{NO}_3^- + \text{NH}_4^+$	-0.94

This low quotient on nitrate is clearly the result of nitrate reduction, a process long ago described by Warburg and Negelein (21), though under very different conditions. A further and most important result is that with both nitrate and ammonia present the quotient is characteristic of ammonia only. The effect of nitrate reduction adds usefulness and precision to the gas exchange quotient as a tool in metabolic studies, for this and other evidence indicates that nitrate is reduced only when ammonia is lacking and only at a rate required by cellular nitrogenous synthesis.

4. At low light intensities the effect of nitrate reduction is a depression of CO_2 uptake. The rate of O_2 evolution is affected little, if at all. In the absence of CO_2 , however, the gas exchange is negligible and there is no evidence of nitrate reduction.

5. Nitrate reduction also accompanies glucose assimilation in the dark, giving rise to an R.Q. of -1.6 as compared to a value of -1.2 when NH_4^+ is the nitrogen source. The marked difference is entirely the result of an increased rate of CO_2 production during nitrate reduction, the rate of O_2 consumption being entirely independent of the source of nitrogen.

6. The extent of nitrate reduction and subsequent nitrogenous synthesis depends upon the previous history of the cells. This

¹ As used herein the quotient of gas exchange, or assimilatory quotient, or respiratory quotient designate the CO_2/O_2 ratio. This value is almost always negative. For convenience, however, the quotients will be called high or low according to their numerical value only.

behavior can be described with reference to Figure 17.1. The cells for these experiments were grown at 40 f.c. under conditions such that they were growing freely and with no limitation save their rate of carbon assimilation. Consider also cells exposed to a photosynthesis-saturating light intensity for a period of three hours. At this high intensity they can produce oxygen about four times as fast as at 40 f.c., at least in the usual short-time experiments, but their rate-of-growth increase is small. Their metabolism has become "growth-bound." And consider still a third type, cells which have been starved aerobically in the dark for several days.

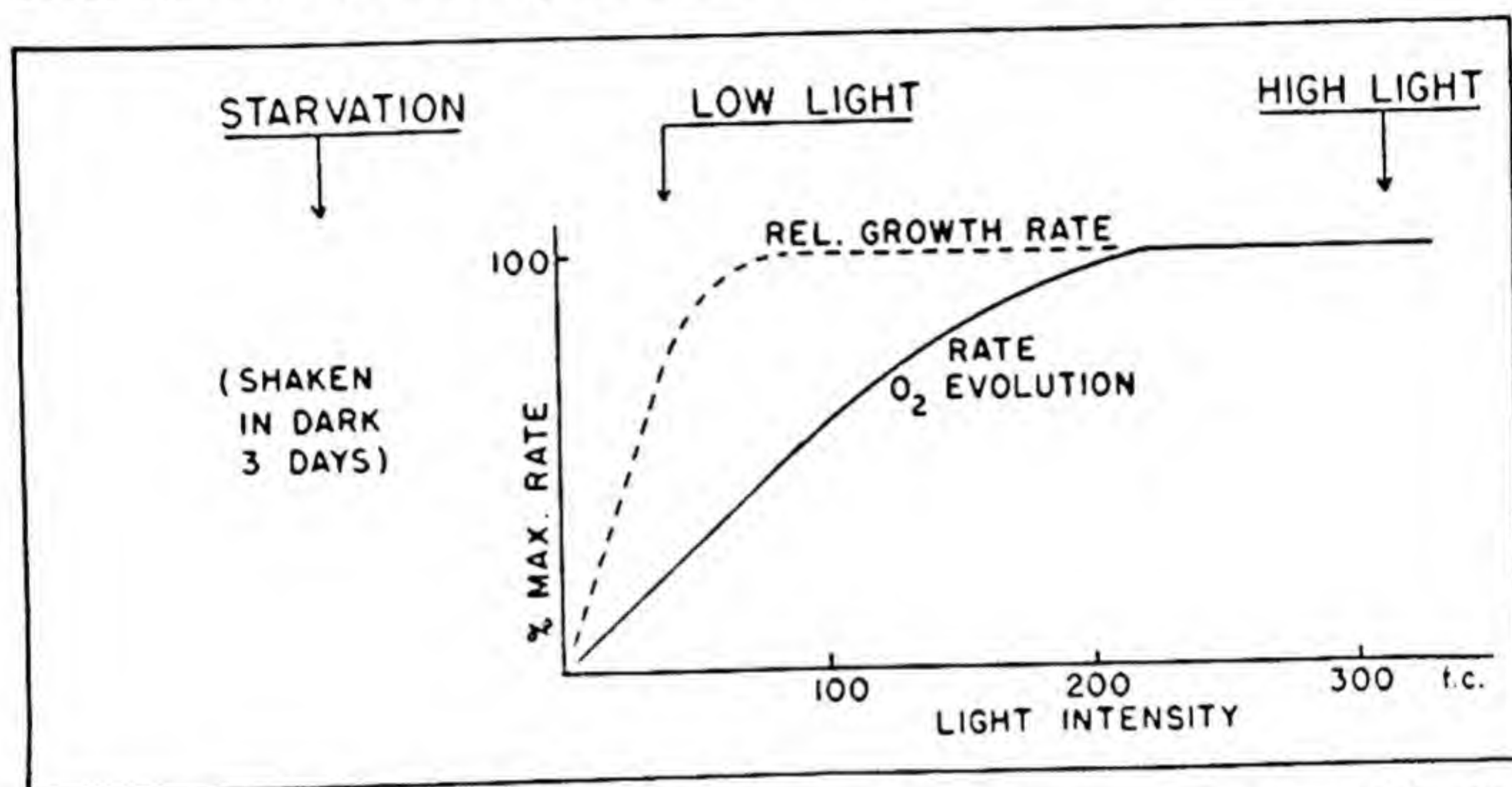


FIG. 17.1—Description of the three types of cell history cited in Table 17.2. Curves for relative growth rate and rate of oxygen evolution are idealized from previously published curves (13).

The three types of cells indicated in Figure 17.1 have been studied under conditions such that the effects of nitrate reduction on the CO_2/O_2 quotient will indicate the extent of nitrogenous synthesis. The results are summarized in Table 17.2. A quotient of -0.68 is characteristic of the cells under the low light intensity at which they have been cultured. At high light intensity the quotient of -0.88 indicates proportionately less nitrate assimilation and a closer approach to carbohydrate synthesis alone. Here is the conventional quotient of -0.9 so often reported. But if, after three hours exposure to a saturating light intensity, the cells are returned to a low light intensity, their low quotient of -0.40 indicates a rapid nitrate reduction and nitrogenous synthesis. Conversely, if the cells are starved, the high quotients approximating complete synthesis to carbohydrate are observed at either high or low light intensity.

A simple explanation suggests itself. The normal or reference metabolic condition is that at low light intensity where there is no

restriction on the conversion of photosynthate into complete cellular materials. On starvation (as will be shown elsewhere) there is a pronounced carbohydrate depletion; to obtain normal growing cells, a rapid carbohydrate synthesis must occur to restore a normal carbohydrate/nitrogen balance. At high light intensity there occurs an overabundant carbohydrate synthesis and on return to normal conditions a rapid nitrogenous synthesis takes place. All of these explanations may be derived also from parallel studies on the effects of nitrogen source and cell history on the R.Q. of glucose assimilation in the dark.

TABLE 17.2
EFFECT OF CELL HISTORY ON THE CO_2/O_2 QUOTIENT IN KNOP'S SOLUTION
CONTAINING NITRATE

Previous History of the Cells	CO_2/O_2 Quotient		Carbohydrate/Nitrogen Ratio (inferred)
	In low light	In high light	
Growing at low light.....	-0.68	-0.88	Normal
Exposed 4 hours to high light.....	-0.40	> Normal
Starved 3 days in darkness.....	-0.91	-0.96	< Normal

It is clear that in *Chlorella* the accumulating products of photosynthetic metabolism may vary greatly with the previous history of the cells. This is a discovery not at all surprising in view of the well-known metabolic variability found within any one of numerous bacteria and fungi. The results have obvious bearing upon the interpretation of data obtained from *Chlorella* with relation to quantum efficiency measurements, the light intensity curve, and the search for intermediates. That the data also have importance of their own will be demonstrated in the subsequent discussion. Up to this point, nitrogen assimilation (as evidenced by nitrate reduction) has been considered only in relation to what has been termed over-all or photosynthetic metabolism. There remains the important question whether the nitrogen assimilation is intimately related to photosynthesis or whether it is merely a part of subsequent respiratory processes.

THE RELATIONSHIP OF NITROGEN ASSIMILATION TO PHOTOSYNTHESIS

While there have been occasional suggestions of nitrogenous synthesis within the photosynthetic process, a much more common viewpoint has held that photosynthesis is exclusively concerned with carbon assimilation. The work of Burström on wheat and some of

the present results on *Chlorella* demand reconsideration of this viewpoint.

NITRATE ASSIMILATION IN WHEAT

Burström (1, 2, 3) has presented quantitative data on carbon and nitrogen assimilation in young, excised wheat leaves. In twenty-

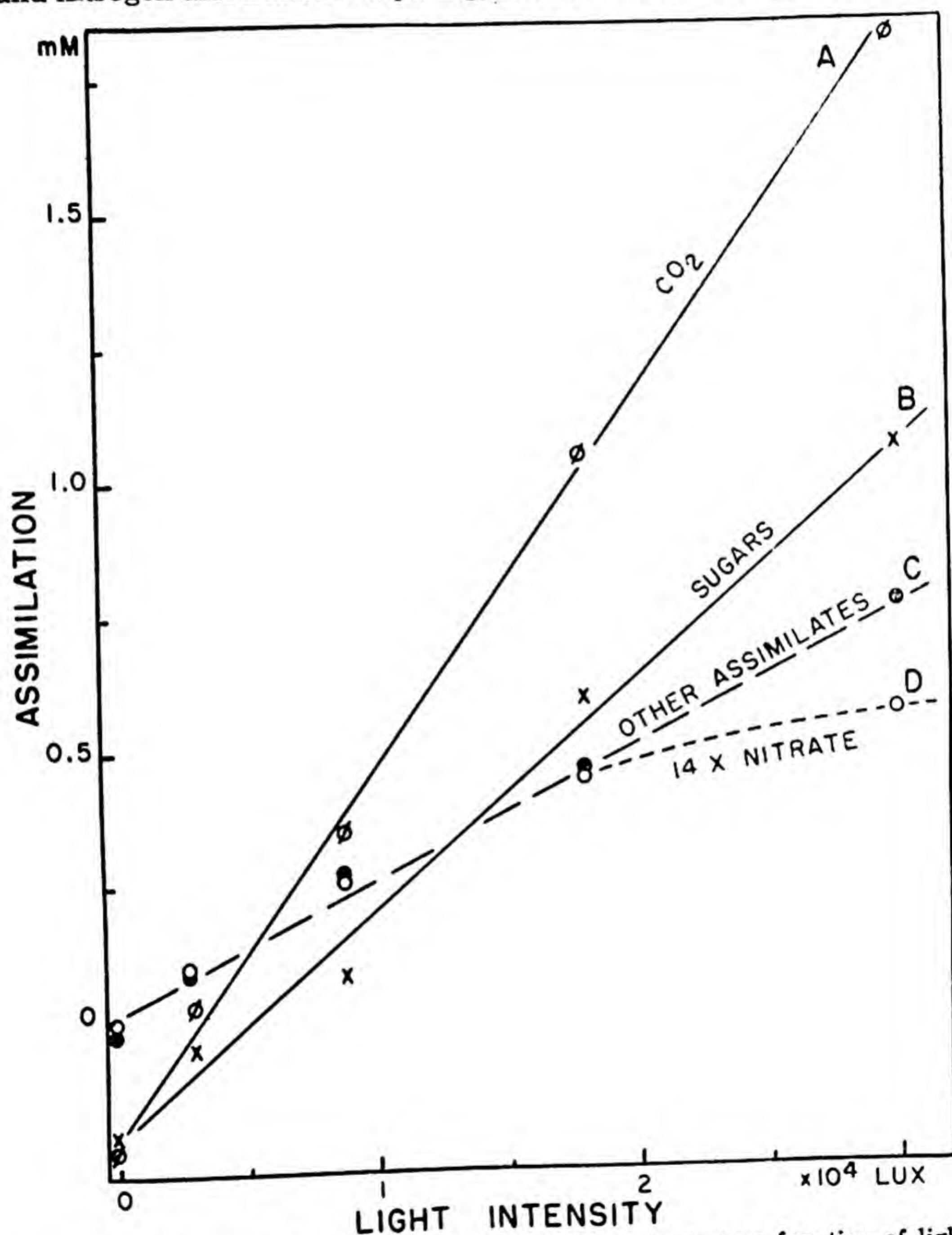


FIG. 17.2—Assimilation in excised young wheat plants as a function of light intensity; data of Burström (2). Assimilation of CO₂ and the production of sugars and "other assimilates" is expressed in millimols carbon per gram lamina-fresh-weight per 24 hours; nitrate assimilation is expressed as 14 \times millimols nitrate disappearing from the plant, per gram lamina-fresh-weight per 24 hours.

four hour experiments under various light intensities he determined the CO_2 taken up, the sugars (hexoses plus sucrose) produced, and the decrease in nitrate content. The salient points of his argument may be considered with reference to Figure 17.2. In darkness there is no appreciable decrease in nitrate and the CO_2 produced can be accounted for almost entirely in terms of the decrease in sugars. Up to 30,000 lux the CO_2 uptake (curve A) and the production of sugars (curve B) were proportional to light intensity. Of the CO_2 assimilated, however, only a fraction could be recovered as sugars; the remainder was labeled "other assimilates" or "CN-assimilates" (curve C). Up to 18,000 lux the production of "other assimilates" was proportional to the decrease in nitrate (cf. curves C and D). The C/N ratio in the "other assimilates" was 14 in this experiment. In a parallel experiment under severe CO_2 limitation the rate of sugar decrease in light was the same as in darkness and was completely independent of light intensity.

Burström's data rule out any explanation of nitrate assimilation in terms of light effects on permeability or respiration, and make any special photochemical process seem most unlikely. The whole argument becomes possible because of the characteristic of the wheat leaf that it will not assimilate nitrate on respiration of sugars alone. An intimate connection between nitrogen assimilation and photosynthesis is required.

In turning to *Chlorella* one finds an experimental organism having a remarkably high nitrogen content and therefore particularly suited to study of nitrogen assimilation. There is at the same time a new difficulty. In contrast to wheat, *Chlorella* can rapidly assimilate either ammonia or nitrate by purely respiratory mechanisms in the dark. The problem here is to find those conditions, if any, under which nitrogen assimilation is directly dependent on photosynthesis and cannot be accounted for by respiratory mechanisms. Three such cases have been found.

EFFECT OF LIGHT INTENSITY ON THE CO_2/O_2 QUOTIENT OF CHLORELLA DURING NITRATE ASSIMILATION

If nitrate reduction and assimilation are completely respiratory processes, this should be demonstrable by studying the effect of light intensity at low (light-limiting) intensities. From the data of Table 17.2 it is seen that both cells grown at low light intensity and cells exposed to high light intensity may show by their quotients a rapid nitrate reduction when studied at low light intensities. Effects of varying low light intensities have been investigated for both types of cells and are presented in Table 17.3. Cells grown at

an effective intensity of about 130 fc. (relative growth rate, 0.82 per day) were used in lieu of cells exposed briefly to high light intensity.

Cells grown at high light intensity have a high rate of dark respiration and a high R.Q. (~ -1.6), indicating that they are continuing a rapid nitrate assimilation at the expense of accumulated photosynthetic products. The apparent photosynthetic quotient is highly variable with light intensity, and may even reach an

TABLE 17.3
GAS EXCHANGE OF CHLORELLA IN DARKNESS AND AT LOW LIGHT INTENSITIES*†

Light Intensity fc.		Cells Grown at High Intensity			Cells Grown at Low Intensity		
		O ₂	CO ₂	CO ₂ /O ₂	O ₂	CO ₂	CO ₂ /O ₂
60	Light.....	4.13	-1.98	-0.48	7.14	-4.90	-0.69
	Dark.....	-2.09	3.38	-1.32	1.78
	Photosynthesis‡	6.22	-5.36	-0.86	8.46	-6.68	-0.79
45	Light.....	3.08	-0.97	-0.32	5.35	-3.19	-0.60
	Dark.....	-2.05	3.23	-1.47	2.07
	Photosynthesis‡	5.13	-4.20	-0.86	6.82	-5.26	-0.77
35	Light.....	1.05	0.69	+0.65	3.43	-2.08	-0.61
	Dark.....	-2.05	3.23	-1.47	1.98
	Photosynthesis‡	3.10	-2.54	-0.82	4.90	-4.06	-0.83

* All rates are given in cmm. gas/cmm. cells/hour.

† Suspending fluid: Knop's solution (containing nitrate) at pH 4.5.

‡ "Photosynthesis" designates the result obtained by subtracting the gas exchange in the dark from that in the light.

anomalous positive value indicating both CO₂ and O₂ evolution. On correcting for the dark respiration, the corrected photosynthetic quotient becomes remarkably constant. In such cells the nitrate reduction can be explained principally, or perhaps entirely, in terms of the dark respiration. The low quotient seen in Table 17.2 for cells exposed to high light intensity is, therefore, not necessarily a characteristic of photosynthesis at all.

Cells grown at low light intensity, on the other hand, have a lower rate of dark respiration and a lower R.Q., indicating, as other experiments have shown, little, if any, nitrate reduction. Here the apparent photosynthetic quotient shows comparatively little variation. The corrected photosynthetic quotient is also relatively stable, and at a low value. Here the process of nitrate reduction appears closely related to the photosynthetic mechanism.

The often-postulated photorespiration might be invoked to explain this phenomenon. Any direct effect of light on respiration of *Chlorella* appears most unlikely (cf. 14). If photorespiration is used to describe an increased rate of oxidative processes arising from accumulating photosynthates, then, unless the photosynthates be required to be carbohydrate, the explanation may be the same as the above with only a difference in terminology. See the discussions below.

EFFECT OF NITROGEN-DEFICIENCY ON THE CO₂/O₂ QUOTIENT IN CHLORELLA

A rapid nitrogen assimilation may also be induced in *Chlorella* by establishing a nitrogen deficiency. If cells are exposed to a high light intensity (~ 300 fc.) and 4 per cent CO₂ for four hours while immersed in a nitrogen-deficient medium, then on return to a medium containing nitrate they exhibit a nitrate reduction so rapid that its effect on the gas exchange quotient may be observed even at light-saturating intensities for photosynthesis. This is a great advantage since the contribution of photosynthesis to the total gas exchange is now some ten to twenty times greater than that of the dark respiration. Representative data from two of a series of experiments are presented in Table 17.4.

TABLE 17.4
GAS EXCHANGE DURING NITRATE ASSIMILATION BY NORMAL AND NITROGEN-DEFICIENT CHLORELLA*†

Expt.		Before Exposure			After Exposure		
		Normal Cells in Knop's (+NO ₃ ⁻)			N-Deficient Cells in Knop's (+NO ₃ ⁻)		
		O ₂	CO ₂	CO ₂ /O ₂	O ₂	CO ₂	CO ₂ /O ₂
A	Light.....	42.5	-36.5	-0.86	39.0	-28.7	-0.74
	Dark.....	-1.4	1.8	-1.3	-2.0	3.2	-1.6
	Photosynthesis ‡....	43.9	-38.3	-0.87	41.0	-31.9	-0.78
B					N-Deficient Cells in Knop's minus NO ₃ ⁻		
	Light.....				31.6	-31.4	-0.99
	Dark.....				-1.4	1.4	-1.0
	Photosynthesis ‡....				33.0	-32.8	-0.99

* Measurements made before and after a four-hour exposure to high light intensity in Knop's solution minus nitrate. Gas exchange measured in darkness and at photosynthesis-saturating light intensity in Knop's, with and without nitrate.
† All rates are given in cmm. gas/hour/cmm. cells (referred to the cell volume before exposure).
‡ "Photosynthesis" designates the result obtained by subtracting the gas exchange in the dark from that in light.

It is seen that, accompanying photosynthesis in a nitrogen-deficient medium, *Chlorella* develops a completely carbohydrate metabolism with a quotient of unity (Experiment B). Note, however, that these are short-time experiments. It is quite likely that in longer exposures in nitrogen-deficient media considerable lipid materials may be produced (cf. 19). On the return to a nitrate-containing medium (Experiment A) the cells exhibit a remarkably low quotient at high light intensity. In order to account for the nitrate reduction here in terms of respiration one would have to postulate an increase in the respiratory rate of at least five times. Such an explanation appears most unlikely, and it seems much more reasonable that the reduction of nitrate is here intimately geared to the photosynthetic mechanism.

ASSIMILATION OF AMMONIA DURING RESPIRATION AND PHOTOSYNTHESIS

In the preceding discussion no distinction has been made between nitrate reduction and nitrogen assimilation. In Knop's solution at pH 4.5 nitrate is reduced only in the absence of ammonia. It is inferred that reduction of nitrate to the level of ammonia is a first step in nitrogen assimilation which does not take place if ammonia is already present. There is also experimental evidence bearing upon assimilation of ammonia. When ammonia is added to a respiring or photosynthesizing suspension in Knop's solution, the gas exchange quotient immediately changes to a value closer to -1.0 ; when the ammonia is consumed there is another sharp break in the quotient. Such behavior allows estimation of the time required by a unit quantity of cells to assimilate a small amount (e.g., 1μ mol.) of ammonia in light and in darkness. A similar procedure has been used to determine the effect of light and photosynthesis on the rate of glucose assimilation (14).

The technique has been applied to nitrogen deficient cells. In practice only a single flask is required. The sharp breaks in the CO_2/O_2 quotient then show up as breaks in the curve obtained by plotting manometer readings of pressure against time. A typical experiment is shown in Figure 17.3. It may be shown also that a second addition of ammonia gives rise to a new break in the curve and that the time required for utilization is approximately proportional to the quantity of ammonia and inversely proportional to the quantity of cells. For nitrogen deficient cells it is necessary to add glucose for the dark experiment in order to obtain sharpness in the second break. Otherwise, for reasons not yet clear, the completion of ammonia uptake is noted only as a gradual transition to zero pressure change, and no return to the high quotient of nitrate reduction is observed. For the present problem, however, the

addition of glucose is an advantage since it approximately doubles the respiration rate of these cells without affecting the R.Q. It will be seen from the data of Table 17.5 that the utilization of ammonia is about two and a half times as rapid during photosynthesis as during glucose-enhanced respiration. It is clear that the

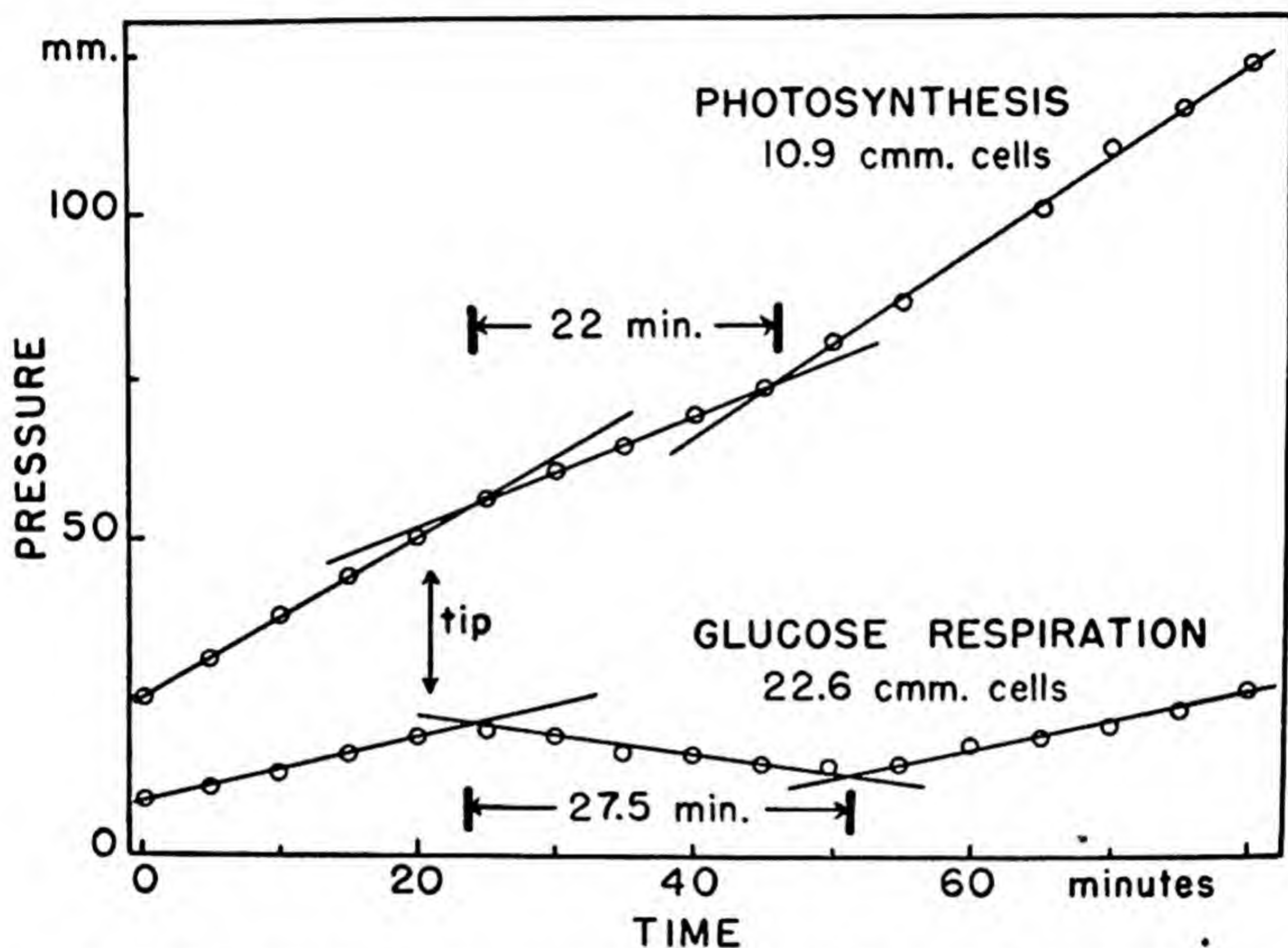


FIG. 17.3—Method of estimating the rate of NH_4^+ assimilation by *Chlorella* during photosynthesis and during glucose respiration. The breaks in the curves are caused by changes in the CO_2/O_2 quotient accompanying the transitions from NO_3^- to NH_4^+ to NO_3^- assimilation. The gas exchange, as indicated directly by the manometric readings, is used only to locate the end points of NH_4^+ assimilation. Experimental details: Respiration in darkness in the usual respiration-type vessel with 2.75 ml. Knop's solution at pH 4.5 and 14.98 ml. air. Photosynthesis at 700 fc. tungsten illumination in a vessel with a flat bottom 10 cm^2 in area and with one sidearm; 6.50 ml. Knop's solution at pH 4.5 and 13.35 ml. of 4 per cent CO_2 in air.

assimilation of ammonia, as well as the assimilation of nitrate, is intimately related to the photosynthetic process.

RECONSIDERATION OF THE PHOTOSYNTHETIC PROCESS

The demonstration that nitrogen assimilation may have an intimate relation to photosynthesis is important in relation to certain requirements which it places upon the photosynthetic mechanism. These will now be considered in a fashion intentionally somewhat

speculative. It is not implied that complete proof has been given of the participation of nitrogen assimilation in photosynthesis. The present data are scanty and in time may become explicable in other ways. It is intended only that by consideration of the implications, the importance of the problem may be made clear.

TABLE 17.5
UTILIZATION OF AMMONIA BY NITROGEN-DEFICIENT CHLORELLA *

Expt.	Glucose Respiration in Darkness				Photosynthesis			
	NH ₄ ⁺ μmols	Cell quantity cmm.	Time min.	Rate μmols/hr. /cmm.	NH ₄ ⁺ μmols	Cell quantity cmm.	Time min.	Rate μmols/hr. /cmm.
A	0.5	24.0	14	0.09	1.0	12.5	21.5	0.23
	1.0	24.0	24.5	0.10	1.0	6.25	46	0.21
	2.0	24.0	46.5	0.11	1.0	12.5	21	0.23
	0.5	12.0	23	0.11	1.0	12.5	23	0.21
	1.0	12.0	42	0.12	2.0	12.5	35	0.27
B	1.0	24.9	20	0.12	1.0	12.0	18	0.28
	1.0	24.9	22	0.11	1.0	12.0	17	0.29
	1.0	24.9	22.5	0.11	1.0	12.0	15	0.33
C	1.0	22.6	27.5	0.10	1.0	10.9	21.5	0.26
	1.0	22.6	29	0.09	1.0	10.9	20	0.27
					1.0	10.9	22.5	0.25
	Average			0.11			0.26

* The cells had been exposed to 400 fc. illumination and 4 per cent CO₂ in Knop's minus-nitrate for 4-5 hours and were then transferred to complete Knop's solution. The experimental method is indicated in Figure 17.3.

A serious difficulty faces all attempts to specify the end product of photosynthesis ([CH₂O] in equation [1]). Photosynthesis cannot be isolated from other metabolic processes in any simple or direct manner. As a result, two points of view are tenable in inferring the nature of the product from the observed gas exchange: (a) the product is written (CH₂O) only as a simplifying approximation; or (b) the product (CH₂O) actually represents carbohydrate, and any deviations from the required gas exchange can be attributed to respiratory processes. The implications of these two viewpoints are quite different.

Carbohydrates are the only compounds of the CH₂O state of reduction found to accumulate in most plants. While it might broaden the argument to label such compounds as acetic acid or formaldehyde as "carbohydrate," from the standpoint of comparative biochemistry this practice has no justification. Substances are related biochemically not by their energy content nor by their

state of reduction, but by the pathways of their interconversions. As used herein, carbohydrate denotes those saccharides which do occur in the plant cell.

That carbohydrate is the general photosynthetic product is an attractive hypothesis. Carbohydrates accumulate in large quantities in higher plants. Carbohydrate is a common substrate for respiratory processes. In the algae carbohydrate may be used in the dark as the sole source of carbon for cellular synthesis. And, perhaps most important of all, the specification of carbohydrate completely defines the end point of photosynthesis. This is a great convenience in any theory based on kinetic data. It has led, for instance, to the neat postulate of eight photochemical steps, equivalent to two times the number of electron transfers necessary to reduce CO_2 to carbohydrate (8).

That carbohydrate is the general photosynthetic product is an hypothesis conveying upon photosynthesis a uniqueness from beginning to end. Photosynthesis then could merge with the rest of metabolism at only one point, the carbohydrate product. The earlier supposed requirement of a four-quantum process, based upon the data of Warburg and Negelein (22), did indeed require such a special physical mechanism. The more recent and extensive measurements of quantum efficiency now indicate a ten- or twelve-quantum process and no longer preclude the possibility of intermediates by which photosynthesis may be meshed with the rest of metabolism. Yet there has been hesitancy to give up the concept of photosynthesis as a unit process. Cogent arguments have been presented for the unit-process concept (cf. 10). But there are no arguments so straightforward and no theories so complete in detail that they preclude investigation of the alternative viewpoint.

An alternative interpretation of present data is that the product of photosynthesis is written (CH_2O) merely as a simplifying approximation and meaning "a reduction product of carbon dioxide." There are also attractive features in this interpretation. It allows the possibility of different photosynthetic products in different plants and the possibility of variation in the products in any one plant depending upon metabolic conditions. It is compatible with a concept of photosynthesis as a process made up of a series of reactions identical or biochemically analagous to the known step-reactions of intermediary metabolism. It is allied with the idea that photosynthesis may become merged with the metabolic stream at a point much earlier than the formation of a single final product.

There have been frequent suggestions that the increasing knowledge of heterotrophic metabolism might provide explanations of

various portions of the photosynthetic process (e.g., 6, 12, 16, 17). The merit of all such suggestions lies in their possible usefulness; none of them have, as yet, been demonstrated as characteristic of photosynthesis.

The study of photosynthesis has now reached a point at which it becomes critically important to determine whether the product is carbohydrate or is more general in nature, whether photosynthesis is a unit process or a metabolically meshed process. The intimate relation between nitrogen assimilation and photosynthesis points to the latter interpretations. Passing over the particular problem of nitrate reduction, since it is not well understood in any organism (cf. 4, 5), consider the known starting point for assimilation of ammonia. Very generally oxalacetic acid and α -ketoglutaric acid are aminated as the first steps in amino acid synthesis. If oxalacetic and α -ketoglutaric are likewise the starting points for nitrogen assimilation in *Chlorella*, then all of our observations become understandable in the proposal that these common metabolic intermediates are also intermediates in photosynthesis. Should this be demonstrated, then a meshing of photosynthesis and metabolism would be established and the photosynthetic products and metabolic products would become identical.

The author is indebted to Marian L. Cramer for permission to summarize here portions of her work as yet unpublished. And he gratefully acknowledges the stimulating suggestions and criticism of his colleague, Jackson W. Foster.

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18.

Some Remarks on Tracer Researches in Photosynthesis

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A DECADE has passed since tracer methods were first applied to the study of photosynthesis by the late Professor S. Ruben in collaboration with Dr. W. Z. Hassid and the writer (1). These studies, begun with the modest hope of finding efficient and rapid methods for synthesis of labeled carbohydrate, quickly revealed the erroneous nature of past speculations about the chemical nature of photosynthetic intermediates (2, 3, 4). Progress toward a real understanding of the primary photosynthetic reactions involved in the assimilation of carbon dioxide seemed assured. Particularly with the discovery in 1940 of a long-lived radioactive isotope of carbon there was made possible the extension in tracer technique required to bring the full weight of organic and biochemical methodology to bear on the problem of isolation of active photosynthetic principles.

However, the outbreak of World War II, coupled shortly thereafter with the tragic and untimely death of Dr. Ruben, effectively stifled research activities in this direction. Thus, the history of the tracer method in photosynthesis still remains largely the chronicle of the three short years 1939-41. The loss of five years in productive research during the war was compensated to some extent by the remarkable developments leading to an unlimited supply of tracer carbon, so that now the abnormal situation of only one laboratory being in a position to carry on tracer research in photosynthesis no longer obtains. It is heartening to note the entry of a number of research groups into this field.

The present symposium affords an opportunity to evaluate early efforts in terms of the more recent data to be presented here

and elsewhere in this monograph. Some data hitherto unpublished are included to aid in clarifying certain sections of the original papers which appear to have suffered from overcondensation. Throughout this paper, these early articles will be referred to as RHK, after the initials of the investigators given in the first papers.

NATURE OF THE TRACER RESEARCHES WITH THE SHORT-LIVED CARBON ISOTOPE, C^{11}

The bulk of the RHK researches were conducted with the short-lived radioactive isotope of carbon (C^{11} , half-life 21 min.). The small time intervals available dictated a research made up of innumerable short-term experiments designed to provide information on a sharply limited aspect of the chemistry involved. Maximal fixation in a minimal time was desirable. Insufficient time was available for thoroughly adequate characterization of products by the usual methods. The dosage techniques implied the use of relatively small quantities of organisms with consequent necessity for the use of the carrier technique in isolating products. The hazards involved in the use of carrier techniques have been discussed elsewhere (2). Further comment will be deferred to sections dealing with the isolation of specific products.

Few reliable gravimetric procedures exist for the isolation of specific constituents of plant tissue. This is a serious difficulty in application of tracer techniques in which it is required to ascertain specific isotopic content. It was a particular source of trouble in working with C^{11} .

The high intensities of radioactivity employed in some experiments (~ 10 mc) appeared to have no effect on the physiological state of the organisms used. However, radiation dosage limitations were important in limiting the research to problems requiring relatively short exposures to labeled CO_2 . Every effort was made to maintain the organisms under conditions which resulted in maximal photosynthetic activity with a maximal ratio of photosynthesis to endogenous respiration. When the dark fixation reactions were being studied, the organisms were maintained in active photosynthesis until the moment of exposure to labeled CO_2 , except in a few experiments in which the effect of previous incubation in the dark was under investigation.

The advantages inherent in the use of C^{11} may be noted. First, no contamination problem existed. All activity vanished in a short time so that elaborate decontamination, storage, or recovery procedures were not required. Thus, all dosage apparatus could be

designed without requirement that labeled material be conserved and recovered. This factor explains many of the differences between the RHK researches and those performed later with C^{14} . Secondly, the assay procedures were simple because of the penetrating radiation. An important consequence of the penetrating radiation from C^{11} was the possibility of using carrier compounds in quantities up to a gram, thereby simplifying chemical procedures for purification. Thirdly, a large number of exploratory researches could be accomplished in a time much shorter than would be required with other isotopes of carbon. Fourth, the time factor, while a handicap to precise and rigorous chemical elaboration, still allowed sufficient time for accomplishing a large number of conventional chemical procedures. Fifth, the investigator could not succumb to the temptation either to put off work or to start experiments without adequate protocols. In this connection it may be pertinent to quote a remark of Dr. Ruben's: "The half-life of a tracer experiment is proportional to the half-life of the tracer."

THE TIME COURSE OF LABELED CO_2 FIXATION IN THE LIGHT

In the RHK experiments, the standard procedure for determining labeled carbon fixation, adopted after numerous exploratory researches, involved gentle boiling of the cells in 0.1 N acid (HCl, or carrier acids) for one minute. The residue from this treatment was frequently tested for labeled carbonate by addition of unlabeled carbonate, followed by repeated boiling in dilute acid. The test organism for the quantitative determination of carbon fixation was a strain of *Chlorella pyrenoidosa* obtained from Professor R. Emerson.

The total fixed carbon determined in this way was found to increase linearly with time. The rate obtained for CO_2 uptake coincided with the rate obtained from manometric measurements of oxygen evolution within the experimental accuracy ($\pm 5\%$). The medium in some cases was a combined phosphate-bicarbonate buffer, in others, bicarbonate buffer at pH 8.5. The algae were exposed to labeled CO_2 only after a previous incubation of an hour or more during which it was established that the photosynthetic evolution of oxygen was proceeding at a maximal and satisfactory rate. With such cells, exchange due to respiratory fixation was minimized. Such exchange was less than 5 per cent of the CO_2 uptake measured, since the rate of oxygen evolution agreed with the rate of CO_2 uptake to within 5 per cent. No tendency to saturation in CO_2 uptake was observed in periods up to as much as three hours. Erratic results were obtained with old cells or young cells insufficiently equilibrated in the light.

The existence of reversible exchange reactions involved in the photosynthetic process could not have been detected in such experiments. The nature of the proof that such reactions exist will be discussed in a later section. It should be remarked that although no manometric determinations of the photosynthetic quotient were made on algae simultaneously with radioactive pickup experiments, control experiments under closely identical conditions always showed the CO_2/O_2 ratio to be between 0.92 and 1.00.

The uptake of CO_2 in light was found to be inhibited precisely as was over-all oxygen evolution by a variety of agents. There may be added here a number of observations from the RHK work which are of interest in connection with the recent findings of other investigators (5, 6). The acid extractable percentage of the fixed labeled material decreased with time. In 5 minutes, 90–100 per cent could be extracted by the boiling procedure with dilute acid; in 25 minutes, 70 per cent; in 110 minutes, 25 per cent. These results are the average of six experiments; the actual number of such experiments performed was at least sixteen, on the basis of notes available to the writer. Data obtained on the variation in chemical characteristics with time will be quoted later.

Dr. M. B. Allen and the writer (6) performed some exploratory experiments on both *Chlorella pyrenoidosa* (strain from the laboratories of Professors C. B. van Niel and H. A. Barker) and on Professor H. Gaffron's *D* strain of *Scenedesmus*. The extraction procedure was changed from that described in the RHK work to conform to parallel experiments on phosphate equilibration. Four grams (wet weight) of *Chlorella* were divided into sixteen parts to give samples with and without KCN (10^{-2} M) at intervals of 5, 20, 60, and 180 minutes. The cells were suspended in 3 ml. 0.1 M phosphate buffer (pH 7) and 2 ml. 0.1 M $\text{NaHC}^{14}\text{O}_3$ added in each case. The activity of the carbonate assayed 40,550 ct./min. per ml., using a thin-window G-M tube (7). The cells were centrifuged at the appropriate time, washed twice with 0.1 M KCN and extracts prepared by freezing in liquid air, followed by thawing in 10 per cent trichloroacetic acid (TCA) for 90 minutes. Any traces of labeled carbonate were completely removed by addition of unlabeled bicarbonate and aspiration of air through the suspension during the acid extraction. The cyanide concentration employed (10^{-2} M) lowered the rate of photosynthesis to slightly below compensation without affecting endogenous CO_2 output markedly. Total fixation of CO_2 measured at 180 minutes was 2 per cent that of cells in the absence of cyanide. Typical data obtained are shown in Table 18.1. It appears that the character of the fixation products in *Chlorella* differed markedly in normal photosynthesis and in "photosynthesis" at compensation.

Similar experiments were performed with *Scenedesmus*. In these runs, a cyanide concentration was used (5×10^{-4} M) which reduced the rate of endogenous respiration fourfold without affecting photosynthetic oxygen evolution markedly. No change in

TABLE 18.1
ACID SOLUBILITY OF LIGHT FIXATION PRODUCTS IN CHLORELLA

Time	Percentage Extractable	
	No Cyanide	Cyanide (10^{-2} M)
5 min.....	91	84
20 min.....	80	83
60 min.....	61	94
180 min.....	43	90

experimental procedure was made, except that the washing solutions were 0.02–0.04 M in cyanide. The use of the cyanide wash in all cases was predicated on the desire to inhibit metabolic activity during the washing procedure. The data obtained are shown in Table 18.2. In *Scenedesmus*, a strict parallelism between over-all photosynthetic activity and increase in acid insolubility was maintained. These results taken together with those for *Chlorella* appear to establish that for the light fixation products in normal photosynthesis an inverse relation exists between time and acid solubility.

In the RHK work it was found also that gentle boiling without acid averaged 80–90 per cent as effective as the same treatment

TABLE 18.2
ACID SOLUBILITY OF LIGHT FIXATION PRODUCTS IN SCENEDESMUS

Time	Percentage Extractable	
	No Cyanide	Cyanide (5×10^{-4} M)
5 min.....	72	66
20 min.....	67	64
60 min.....	41	..
170 min.....	21	29

with 0.1 N acid in recovering soluble fixation products. In one experiment the following extraction procedures were employed; cold acid treatment, cold treatment in the presence of a carrier aldehyde mixture, cold extraction in an acid solution containing 2,4-dinitrophenyl hydrazine, cold alcohol treatment, and finally, extraction with boiling water. In no case were any of the agents mentioned more than 20–30 per cent as effective as hot water. Other efforts involved grinding the cells with powdered glass at

room temperature in saline solution as well as in the presence of various detergents. Again, only 20–30 per cent extraction was noted. In the later experiments quoted (6), using C^{14} , extraction efficiencies comparable to hot water treatment could be achieved with the liquid air freezing treatment followed by thawing in the presence of dilute acid.

THE CHEMISTRY OF THE LIGHT FIXATION PRODUCTS

The RHK experiments showed that no significant preferential fixation occurred in any of the well-known organic constituents of cells tested. These included representatives of all compounds of low molecular weight ($M.W. < 300$). The vapor pressure of the labeled material, whether formed in the first few minutes or after several hours, was very small at $120^{\circ}C$. (1% lost by heating for 1 hour).

The tests were carried out primarily on material fixed in the first few minutes in the light. This material remained in water solution in the presence of typical organic solvents such as ethyl ether, petroleum ether, chloroform, and butyl alcohol, over a wide range of pH. This extraordinary hydrophilic characteristic was first ascribed to the very low concentrations of labeled material encountered. However, the dried residue at $120^{\circ}C$. was also insoluble in organic solvents.

The original RHK papers state that "no activity" was found in the large variety of compounds tested. Actually, the percentage fixed in any characteristic precipitate was always detectable, varying from 0.05 per cent to 15 per cent. However, high activities were invariably associated with precipitates which were not well-defined or crystalline. In every instance where recrystallization or purification by solvent extraction was followed, the specific activity fell continuously with repeated purifications. In the case of formaldehyde in one experiment, the characteristic 2,4-dinitrophenyl hydrazone decreased in activity from 5 per cent that fixed in the total cells to a final activity of 0.003 per cent, after three recrystallizations in presence of carrier aldehyde mixture. The inability to properly characterize certain precipitates, such as those obtained by precipitation with chromate or sulfuric acid (tannins), phenolic carboxylic acids, etc., rendered unsuccessful the attempt to test the possibility that such compounds actually partake to an appreciable extent in primary fixation products.

It should be emphasized that in most of the characterizations used the general procedure involved recrystallization from one solvent. Such a procedure is known to be unreliable (8) and it was not considered as a definitive test procedure in the RHK work. Whenever possible, partition between solvents was employed. Thus, the

presence of alcoholic hydroxyl groups was established by a room temperature, acid benzylation of the active material, using, as carrier, mannitol or glucose, the benzoyl ester being extracted from the aqueous layer with chloroform at neutral or alkaline pH. After a number of extraction cycles, the product specific activity remained constant at a value varying from 10 to 24 per cent of the total soluble fixed material.

The presence of active carboxyl was shown by appearance of active carbonate after dry distillation of a precipitate obtained by barium-alcohol precipitation of carrier acid mixtures. A very large number of runs was made to establish this point. The barium (or lead) precipitates remained at constant specific activity through at least five reprecipitations, the fixation corresponding to 35–40 per cent of the total soluble fraction fixed in a few minutes. The exact amount of active carboxyl could not be ascertained because the proper decarboxylation conditions could not be predicted without exact chemical knowledge of the particular carboxylic product. However, under conditions which gave 80 per cent decarboxylation of the carrier acids, some 7–10 per cent of the fixed material was decarboxylated. The amount appearing in the carboxyl group fell rapidly with time of photosynthesis. One RHK experiment is available which shows 7 per cent carbon monoxide formed by cold sulfuric acid digestion of *Chlorella* incubated a few minutes in $C^{11}O_2$ in the light. This result supports the notion of an α -hydroxy or α -keto carboxylic acid as an intermediate.

Recently (6), the light products from *Scenedesmus* incubated one hour in 0.1 M labeled bicarbonate, in the presence of 5×10^{-4} M KCN (to minimize respiratory fixation) have been extracted on a scale sufficient to prepare approximately 300 mg. of the insoluble barium salt without added carrier. This material was heated at $250^\circ C$. for $2\frac{1}{2}$ hours in a stream of nitrogen (conditions identical with the RHK researches and probably optimal for most hydroxy acids). Of the activity fixed in the barium salt 12 per cent appeared as carbonate. In the RHK work, no volatile ketone was formed. However, no effort was made to test the residue for a non-volatile ketone. The fact that no volatile ketone formed was used to support the notion that a high molecular weight acid was involved in the decarboxylation. However, this interpretation was based on the assumption of a fatty acid without many hydroxyl groups. Hence, in this later work, the residue was examined and found to give an appreciable fraction of the activity in a well-characterized 2,4-dinitrophenyl hydrazone. However, the yield of ketone corresponded to only one-third that to be obtained from a simple decarboxylation. Some experiments involving high temperature decarboxylation in

mineral acid solutions have also been performed and indicate qualitatively the presence of carboxyl groups.

It would add nothing to this discussion to review in detail the many experiments performed in the RHK work to elaborate the chemical nature of the light product. It suffices to state that between 500–1,000 experiments were performed in an effort to characterize the fixation products. The conclusions to be drawn were either that the fixation products were not related to any of the commonly known plant or animal constituents, or that the fixed carbon was distributed, even in a few minutes, homogeneously over a small infinity of products, the total fixed in any one product being too small to detect. The results appear to be borne out both quantitatively and qualitatively by later work both in the writer's laboratory and elsewhere. Reports from the Chicago group (5) substantiate the conclusion reached in the RHK researches that the chemical behavior of the compound indicates a high degree of hydroxylation and carboxylation in a complex polyfunctional compound(s).

"DARK" FIXATION OF CO₂

In the original RHK papers, evidence was submitted in support of a thesis that the CO₂ fixed reversibly in the absence of light was associated with photosynthesis. It was shown that the dark fixation product could be equilibrated isotopically with exogenous carbon dioxide by alternate pumping and re-introduction of gas. The dark product remained largely water soluble during the whole incubation period (40 min.) in contrast to the behavior of the light products. Like the light products, the dark fixation products could not be characterized chemically. They reacted in a manner similar to the light product on benzylation but gave a greater percentage of carboxyl on decarboxylation.

It was pointed out in the RHK papers that the yield of carboxyl approached that for complete decarboxylation of a straight-chain fatty acid. It was assumed that most of the dark fixation halted at the carboxyl stage. Precise interpretation of the carboxyl yield figures could not be given because of dependence of the maximal decarboxylation yield on the kind of organic acid treated. Because it was expected that the intermediate, called RCOOH, might contain numerous hydroxyl groups, the theoretical yield of 50 per cent for a straight-chain fatty acid meant little as a norm. Thus, while barium butyrate at its optimal temperature decarboxylates completely to yield 50 per cent carbonate, barium salts of acids like pyruvate or gluconate give variable yields from 50 to 75 per cent of theoretical (6).

The dark fixation in *Chlorella* was inhibited quantitatively in

the same degree as photosynthetic oxygen evolution by a variety of agents (cyanide, phenyl-urethane, ultraviolet light). The relative degree of dark fixation was higher in *Chlorella pyrenoidosa* than in *Chlorella vulgaris*, under conditions in which it had been ascertained that the latter organism possessed a less active photosynthetic apparatus while exhibiting the same rate of respiratory utilization of endogenous reserves. This experiment, as well as some with etiolated barley, demonstrated the dark fixation to be independent of chlorophyll concentration.

The same chemical tests were conducted on the dark fixation products as those reported for the light products, and similar results were obtained. However, because of the much lower yield of product fixed in the dark, it was not possible to set a limit lower than 10 per cent of the total fixed in radioactive heterotrophic assimilative products such as the simple dicarboxylic acids. Small activities were found in molecules such as succinic acid, but because of the low level of fixation, the absolute activity was too small to make possible a rigorous demonstration of the reality of fixation in such molecules. It was certain that the major portion of the dark products could not be accounted for in terms of known plant constituents, just as in the case of the light products. Determinations of sedimentation and diffusion constants, to be discussed briefly later in this report, indicated that both dark and light products were considerably larger molecules than any of the known respiratory intermediates and similar to each other in respect to molecular physical constants.

Later experiments (9) on both *Chlorella* and *Scenedesmus* have agreed with the RHK results on cyanide inhibition in exhibiting a parallelism between dark CO_2 -fixation reactions and over-all photosynthetic activity, provided the cells are maintained under normal photosynthetic conditions. It has been shown that this parallelism breaks down when the algae are maintained under fermentative conditions.

Recently (6) the acid solubility of the dark fixation products has been investigated in a manner identical with that described previously for the light products. The solubility data are given in Table 18.3. In *Chlorella*, a decrease in solubility is observed, reminiscent of the behavior of the light products. The solubility behavior in the dark without cyanide is much like that in the light with cyanide. In *Scenedesmus*, dark fixation solubility data appear to exhibit constancy with time, with or without cyanide.

All of the data cited can be fitted into a scheme based on the original hypothesis of Ruben and Kamen that the primary fixation step involves reversible carboxylation followed by reduction with

intermediate hydrogen donors formed in light reactions. However, the precise nature of the relation between dark and light products remains in doubt. A weakness in much of the data on inhibitors is the absence of data gathered simultaneously on the gas balance (manometric) of the algal systems in the presence of the inhibitors. The interpretations of the differential inhibition observed are based primarily on the assumption that normal photosynthetic quotients are maintained.

The investigations of the Chicago group (5) have confirmed the RHK observations that the dark fixation products can be equili-

TABLE 18.3
ACID SOLUBILITY OF DARK FIXATION PRODUCTS

Time	Percentage Extractable			
	Chlorella		Scenedesmus	
	No Cyanide	Cyanide (10 ⁻² M)	No Cyanide	Cyanide (5 x 10 ⁻⁴ M)
5 min.....	94	99	70
20 min.....	90	74	70
60 min.....	82	94	79	83
180 min.....	69	88	77	72

brated with external CO₂. This work has also demonstrated that the light reaction products cannot be brought into reversible equilibrium with CO₂ in the dark, a finding in essential agreement with the postulate of the irreversibility of the light fixation. From this fact, it might be inferred that there is no connection between light and dark fixation products. Another fact cited in this connection is the very low level of fixation in dark products. RHK observed fixations after twenty minutes corresponding, at most, to 1-2 per cent of the fixation observed in the light in a few minutes. Similar results are reported by the Chicago group.

It should be remarked that arguments based on magnitude of isotopic concentration are weak on two grounds: (a) there may not be isotopic equilibration; RHK showed that equilibration could be attained only by repeated pumping in and out of CO₂; (b) no data are available on specific isotopic content of either dark or light products. The first objection implies that the actual magnitude of dark-product concentration is at least fivefold greater than that noted by simple exposure to exogenous carbonate. The differential of perhaps a factor of five in magnitude of fixation in dark and light remaining to be accounted for can be ascribed to displacement of

fixation reactions because of removal of dark products in following light reactions. The second objection raises the whole question of procedure in establishing precursor-product relations using isotope labeling methods. It is impossible to make positive statements about any given molecule as a precursor for another without data on the time variation in isotopic content of both molecules. Such data require isolation of the pure dark and light fixation products, an accomplishment still probably far in the future.

Before concluding this section, it is desirable to recall an experiment performed by Ruben and Kamen (4) which bears on the metabolism of the dark fixation products in the light. *Chlorella* cells were allowed to equilibrate with labeled CO_2 in the dark (twenty-minute exposure). The labeled CO_2 was then removed and replaced with unlabeled CO_2 . A sample of the cell suspension was taken at this time and assayed for fixed dark products. A portion of this sample was used to prepare the insoluble barium salt, using the acid carrier mixture. As usual, nearly all the fixed material was acid extractable and barium (alcohol) precipitable. Decarboxylation of the barium salt, prepared with the carrier acid mixture employed in previous characterizations of the dark products, showed that most (80–100%) of the fixed soluble carbon was in the carboxyl group.

The cell suspension with its fixed carbon content was exposed to light in the presence of unlabeled CO_2 for twenty minutes, at the end of which time the cellular suspension was again analyzed for total activity and distribution of fixed products. The total activity was found to have remained unaltered. The expected reduction in acid extractable products was also noted, only 50 per cent of the total fixed activity (75–80% of the soluble activity) being precipitable with barium. Decarboxylation of the barium salts prepared in a manner identical with that used for the dark fixation products showed very little activity in the carboxyl group. Thus the dark fixation product was altered by a twenty-minute exposure in the light in a manner to be expected on the assumption that the carboxylation product formed in the dark could be used as a precursor for the light products. Unfortunately, there exists an alternative explanation—that the dark product was first decarboxylated and then the resultant labeled CO_2 used to form light products. Had this occurred, one might suppose there should have been a marked decrease in total activity of the cells because of dilution of the evolved labeled CO_2 with the large reservoir of unlabeled CO_2 present. However, it is impossible to decide this point because such dilution depends on the degree of equilibration between exogenous and endogenous carbonate.

PHYSICAL CHARACTERISTICS OF THE FIXATION PRODUCTS

A few brief remarks about results bearing on the sedimentation and diffusion constants of the fixed products may be interpolated. The sedimentation of both light and dark products was studied by Ruben, Kamen, and Perry using an opaque type of ultracentrifuge (10) the rotor of which could be halted for sampling. The measurement of molecular weight by the sedimentation method is best suited to very large molecules, such as proteins. However, accurate values have been reported for sucrose (11) using such an apparatus. Sucrose was used to calibrate the ultracentrifuge employed in the experiments by Ruben, Kamen, and Perry.

Six good runs were recorded out of a few dozen attempted. The fixation products from exposure either in light or dark for periods up to twenty minutes exhibited sedimentation velocity constants ranging from 5.7 to 8.6×10^{-14} c.g.s. units. The few values obtained together with the rather large deviations discouraged any effort to correlate sedimentation characteristics with variations in time of exposure to light and dark. The only conclusion warranted appeared to be that the dark and light products as obtained by the acid extraction of *Chlorella* had sedimentation velocity constants not differing by more than 50 per cent and exceeding that for sucrose by a factor of four. The diffusion constants for light and dark material were also identical within the same rather large margin of error, but were very nearly identical with that obtained for sucrose, as determined with the Northrop-Anson technique (12). The diffusion velocity of the fixation products was increased at high ionic strengths. This fact, together with the anomalous, high diffusion velocity compared to the sedimentation velocity, and the chemical evidence for a polyfunctional molecule with many carboxyl and hydroxyl groups influenced Dr. Ruben and the writer to suggest that the fixation products were highly polar asymmetric molecules with a molecular weight which could be calculated to lie anywhere in the range between 700 and 1,500.

The material on which these rough determinations of molecular weight were made was very probably heterogeneous and impure, as no purification other than filtration through a fine filter disk (Jena, No. 4) was made. The percentage of the dry weight of the *Chlorella* extracted by the acid treatment was 6 to 8 per cent. The material on which the Chicago group has been working is reported in the initial steps of their purification as only 2 per cent of the dry weight of their algae (*Scenedesmus*). The final material on which the Chicago group will make molecular weight determinations will probably be at least fifteen times purer, so that whatever

result is obtained can hardly be expected to have a close relation to the values given by Ruben and Kamen for the highly heterogenous material which they were able to obtain.

The sedimentation and diffusion measurements made with C^{11} on the photosynthetic products are interesting because of the very high dilutions encountered. The concentration of material analyzed was in the order of 10^{-5} M.

PHOSPHATE METABOLISM

Much interest attaches at present to experiments on phosphate metabolism in relation to photosynthetic activity. A number of writers have suggested (13, 14) that light metabolism may be correlated directly with phosphorylation, the requisite energy for fixation reactions being provided by ester phosphate bonds. Most recent is the proposal by Lipmann (15) which pictures a mechanism based on model reactions generalized from the experimentally observed reversal of the "phosphoroclastic" split of pyruvic acid. In this scheme, a carboxyl group is phosphorylated and then reductively decarboxylated to a keto acid. The keto acid is then hydrogenated to give a hydroxy acid. The cycle then repeats, beginning with phosphorylation of the new hydroxy acid. In all the theories proposed, light absorption leads to the formation of phosphate esters at the expense of cellular inorganic phosphate, while energy dissipation attendant on CO_2 fixation results in degradation of organic phosphate to inorganic phosphate. In some manner as yet unspecified, the large energy differential in each quantum of absorbed light (~ 50 kg. cal.) is parceled up efficiently into many smaller packets and stored as phosphate bond energy. Whatever the merits of this suggestion, it is apparent that data on phosphate turnover as a function of light and dark metabolism are urgently needed.

A characteristic of cellular metabolism in photosynthetic systems as well as in most cells is the constancy observed in internal composition despite the continual flux of metabolites during biological activity. Thus, the net changes to be detected in P distribution during metabolism are small, rendering the detection of flow of phosphate between various cellular fractions difficult, if not impossible, utilizing gross P analysis. The availability of labeled phosphorus affords an opportunity to check the existence of flow of phosphorus between cellular fractions when no appreciable net change occurs. The problem becomes the characterization of the phosphorus compounds in plants and determination of their turnover during periods of photosynthesis compared to periods of endogenous respiration. No extensive data on the phosphate composition of plants in terms

of phosphate compounds exists. That it differs markedly from the composition found in animal tissues is apparent from both past and present work (14).

As a point of departure, H. Gest and the writer have been using a scheme for P fractionation developed for analysis of P turnover in yeast (16, 17). At this time, our experiments (18) are insufficient in number to warrant conclusions on the relation between photosynthetic activity and phosphorylation. It may be said that the behavior of algae and certain of the purple non-sulfur bacteria resemble yeast in that complete equilibration with exogenous phosphate is not obtained. There appears to be a correlation between the rate of phosphate entry and the rate of photosynthesis. The soluble phosphate (extractable with ice-cold 5% trichloroacetic acid) appears to turn over more rapidly than the residue phosphate. The ratio of turnover in either fraction in light to that in dark is very nearly equal to the ratio of photosynthetic oxygen evolution to respiratory CO_2 evolution, measured manometrically.

The photosynthetic organisms exhibit a marked difference relative to cells like yeast in that they have a significant fraction of labile phosphate (one-third to one-half of the total soluble phosphate) which equilibrates rapidly with exogenous phosphate, and which apparently can be removed from the cells merely by exhaustive washing with ice-cold saline, distilled water, or buffer solutions. A similar phenomenon closely related to this washing behavior can be noted in the reports of a number of investigators (19, 20, 21, 22, 23) using diverse microorganisms. Apparently the significance of the existence of such washable phosphate has not been assessed previously. Cells from which such phosphate has been removed appear to exhibit normal photosynthetic activity, at least as measured by oxygen evolution. Interpretation of turnover data may require analysis of this washable fraction as well as the "basic" phosphate which resists rapid equilibration.

For the present it is intended to study cells in which easily exchangeable phosphate has been removed by washing. The difference in turnover between residual soluble phosphate and residual insoluble phosphate affords the opportunity to establish differential labeling in these two fractions, so that flow of phosphate between residue and soluble portions as a function of photosynthetic activity can be studied.

CONCLUDING REMARKS ON MISCELLANEOUS MATTERS

It is generally agreed that a major hindrance to progress in elucidation of the chemical mechanisms of photosynthesis is the

unavailability of active cell-free extracts in which component enzyme systems can be studied. A most important contribution to this problem has been the development of techniques for isolation of the oxygen-evolving system of chloroplasts (24, 25). A similar result for CO_2 fixation coupled to oxygen evolution or hydrogen transfer has yet to be achieved.

It should be possible, using labeled carbon, to monitor cell extraction procedures so that CO_2 pickup can be ascertained when no net fixation can be observed. Knowledge of the CO_2 acceptor substrate is required and awaits elucidation of the labeled molecules produced from labeled CO_2 .

Some notion of the labile nature of the photosynthetic fixing system can be obtained from results observed using C^{11} (26). The light fixation of C^{11}O_2 was investigated in the following systems: (a) barley leaves dried in high vacuum and suspended in water; (b) barley mush obtained from the Waring blender; (c) *Chlorella* dried in high vacuum and suspended in water; (d) barley juice expressed from an hydraulic press; (e) intact barley and *Chlorella* as controls. The radioactivity fixed in the dried barley in five minutes was only 0.3 per cent of that fixed in the same time by the same quantity of intact plants. The barley juice fixed 0.15 per cent that of the controls, the barley mush, 0.2 per cent. Dark fixation in the dried barley was only 0.02 per cent of the control. In the algae, drying reduced the fixation to 0.92 per cent that found in intact cells.

In other experiments, preparations were made by grinding the cells after freezing at liquid air temperatures. Acetone powder preparations analogous to those reported as successful for extraction of CO_2 fixing system in mammalian tissue (27) have also been made. Results comparable to those cited above were obtained. These observations merely emphasize the notorious fragility of the CO_2 fixation systems in photosynthetic organisms compared with those associated with heterotrophic fixation.

It may be remarked in conclusion that the contribution of the tracer method to knowledge of photosynthesis mechanisms has not been entirely of a negative nature. Experiments with O^{18} -labeled water and carbonate (28, 29) have shown that the oxygen evolved in photosynthesis does not arise to any appreciable extent from carbonate but originates almost entirely from water. A detailed discussion of these experiments has appeared elsewhere (30). The inability to characterize the fixation products is in itself a positive contribution because it supplies an incentive to investigate the chemical nature of the soluble constituents of the lower plants.

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19.

C^{14} in Photosynthesis¹

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A NUMBER of tracer elements have already been used in an attack on the problem of photosynthesis. These include oxygen 18 (1, 2, 3, 4), tritium (5), deuterium, phosphorus 32 (6), and carbon 11 (7). The importance of the carbon isotopes for such a study is, of course, obvious, and with the ready availability of the long-lived carbon 14 since 1945, the study of the path of carbon in photosynthesis has been undertaken in a large number of laboratories in this country.

The method of attack is a straightforward one. First, the plants must be fed labeled carbon dioxide under as wide a variety of conditions as seems feasible, ranging from dark feeding after suitable pretreatments to increasingly long periods of photosynthesis in the presence of radioactive carbon. Included also should be a variation in the dark time following the administration of labeled carbon dioxide in the light. During the courses of these experiments, the kinetics of the total incorporation of the radioactive carbon dioxide should be studied under each set of conditions, following which an analysis of the plant substance is made in order to identify the compounds or substances into which the radioactive carbon has been incorporated. After these have been identified, the distribution of the radioactive atoms within each compound is to be determined.

With these data at hand, it becomes possible to make hypotheses describing the sequence of intermediates through which the carbon passes on its way from carbon dioxide to the various plant constituents. From the effect of the several variables on the nature of the compounds into which the tracer is incorporated and the rate at

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which they appear, it should be possible to determine the relative importance of the proposed steps. It should then be possible to test these hypotheses by the usual methods of biochemistry, such as the administration of synthetically prepared labeled intermediates, the use of various poisons, and, finally, the attempted isolation of the enzymatic and photochemical units from the organized plant cells, leading ultimately, perhaps, to the possibility of the reconstruction of the whole sequence of reactions, each separated from the other.

The bare results of the beginnings of such an investigation have already been reported (8, 9). It is the purpose of this paper to describe the experimental methods that were used to achieve these results, together with a discussion of them. Because the preparation of the biological materials (algae), the radioactive material and its measurement, and the chemical isolations, identifications, and degradations were very much the same for each experiment in which the method of administration and the immediate pretreatment of the algae were varied, these items will be discussed first in general terms. The specific results for each type of experiment will then be presented, followed by a hypothesis based upon these results.

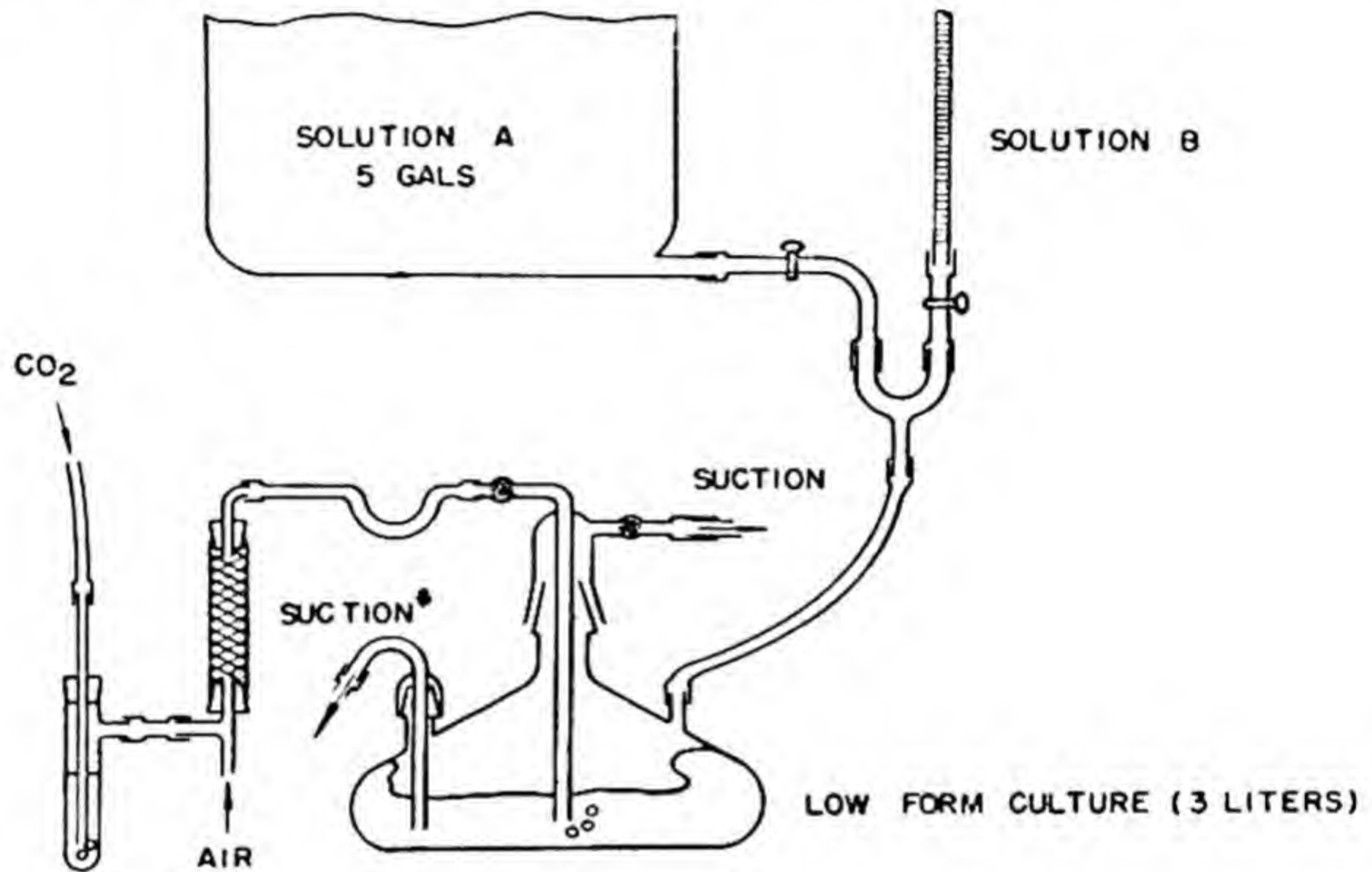
EXPERIMENTAL

Continuous cultivation of Chlorella pyrenoidosa and Scenedesmus D₃.——The algae, obtained through the courtesy of Professor H. Gaffron, Department of Chemistry, University of Chicago, were grown in continuous culture flasks as pictured in Figure 19.1. The flasks were mechanically shaken and a stream of air (1,700 ml./min.) and CO₂ (190 ml./min.) drawn through the flasks. The flasks were illuminated from below by two 100-watt white (4,500°K) fluorescent lights at an intensity of approximately 5,000 lux. The temperature varied from 20°–27°C. The culture solution, made with distilled (glass) water, had the following composition:

KNO ₃	0.005 M	0.506 g./liter
KH ₂ PO ₄	0.001 M	0.136 g./liter
MgSO ₄ · 7 H ₂ O.....	0.002 M	0.493 g./liter
Ca (NO ₃) ₂	0.00025 M	0.041 g./liter
H ₃ BO ₃	0.000028 M	1.43 mg./liter
MnSO ₄ · H ₂ O.....	4.5 x 10 ⁻⁶ M	1.05 mg./liter
ZnCl ₂	3.7 x 10 ⁻⁷ M	0.05 mg./liter
CuSO ₄ · 5 H ₂ O.....	1.6 x 10 ⁻⁷ M	0.04 mg./liter
H ₂ MoO ₄ · H ₂ O	5.6 x 10 ⁻⁸ M	0.01 mg./liter
Fe (NH ₄) ₂ (SO ₄) ₂ · 6H ₂ O.....	1.58 x 10 ⁻⁵ M	6.2 mg./liter
pH 4.5		

All constituents except the Fe (NH₄)₂ (SO₄)₂ were mixed, desig-

nated as solution A, and autoclaved for 20 minutes at 15 lbs. The $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ solution, designated as solution B, was prepared by adding 5.6 g. of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ to one liter of boiling water, previously acidified to pH 2 with 6 N H_2SO_4 , and autoclaved immediately. The acid pH and removal of oxygen prevented the oxidation of ferrous ion during autoclaving. One ml. of this solution was added to 899 ml. of solution A each day to make the final culture solution.



* FOR HARVESTING

FIG. 19.1—Apparatus for continuous culture of algae.

Stock cultures of *Chlorella pyrenoidosa* and *Scenedesmus D₃* were kept on 2 per cent agar slants of solution A and B plus 0.2 per cent glucose. After growth, the slants were stored at 5°C. until used.

Chlorella pyrenoidosa was harvested every 24 hours by withdrawing 900 ml. of culture from the flask. Approximately 1.5 ml. of packed cells (20 min. at 530 G.) were obtained at each harvesting. A refrigerated centrifuge was used to prevent heating of the cells during the centrifugation. When centrifuged without cooling, the cells were heated enough to greatly impair their rate of photosynthesis. *Scenedesmus D₃* was harvested every 48 hours, and approximately 1 ml. of packed cells was obtained per 900 ml. of medium. The growth of the cultures may be determined by placing a calibrated photronic cell in series with a microammeter at the surface of the culture flask to measure the light absorbed by the organisms.

After harvesting, 1 ml. of solution B and 899 ml. of solution A were added to the culture flask. The 100 ml. of culture medium remaining in the flask served as an inoculum for this solution.

The average rate of photosynthesis of cells grown under these conditions was $0.47 \text{ mm.}^3\text{O}_2/\text{mm.}^3 \text{ cells/min.}$ for *Chlorella pyrenoidosa* and $0.43 \text{ mm.}^3\text{O}_2/\text{mm.}^3 \text{ cells/min.}$ for *Scenedesmus D₃*.

One milliliter of packed *Chlorella* cells contained 4.4 mg. of chlorophyll and 0.155 g. of dry matter. One milliliter of packed *Scenedesmus* cells contained 5.5 mg. of chlorophyll and 0.127 g. of dry matter.

Continuous cultures of *Chlorella pyrenoidosa* have been grown for two and one-half months; then the production of mucilaginous material by the organisms caused the cells to adhere to one another and to the surface of the culture flask. These cells settled rapidly and showed a decreased rate of CO_2 fixation. Therefore, the culture was abandoned and a new continuous culture started with a fresh inoculum. *Scenedesmus D₃* cultures started to clump after several months of continuous culture. At this time, a new culture was started using an inoculum from an agar slant.

When the cells were centrifuged, a thin layer of white material appeared on the surface of the packed cells. This material, when examined microscopically, appeared to be debris from dead cells and no bacteria were seen in this white layer.

When solution A was used to prepare the culture medium, the initial pH was 4.5. At the time of harvesting, the culture of *Chlorella pyrenoidosa* had a pH of 7.3 and the culture of *Scenedesmus*, a pH of 6.5. When $(\text{NH}_4)_2\text{SO}_4$ replaced KNO_3 in solution A and CaCl_2 was used instead of $\text{Ca}(\text{NO}_3)_2$, no growth of *Chlorella pyrenoidosa* was obtained. However, when the pH of this medium was changed from the initial value of 4.5 to pH 8.7–9.0 with 1 N NaOH, growth was as rapid as in the corresponding nitrate medium at pH 4.5. During growth in an ammonium medium the pH decreased so that in 24 hours the pH of the culture was between 6.5–7.0. After 48 hours the pH was 3.0 and growth had ceased. Cells transferred from the culture medium at pH 3 to fresh medium at pH 8.7 were unable to grow. When growing continuous cultures of *Chlorella pyrenoidosa* in an ammonium medium, the pH of the culture was determined by drawing a portion of the liquid into a chamber attached to the culture flask which contained micro pH electrodes (see Fig. 19.2). The culture was kept between pH 9.0–7.0 by adding 1 N NaOH at approximately 12-hour intervals.

Barley seedlings.——Barley seedlings were grown in the greenhouse on screen trays over water and freshly cut for experi-

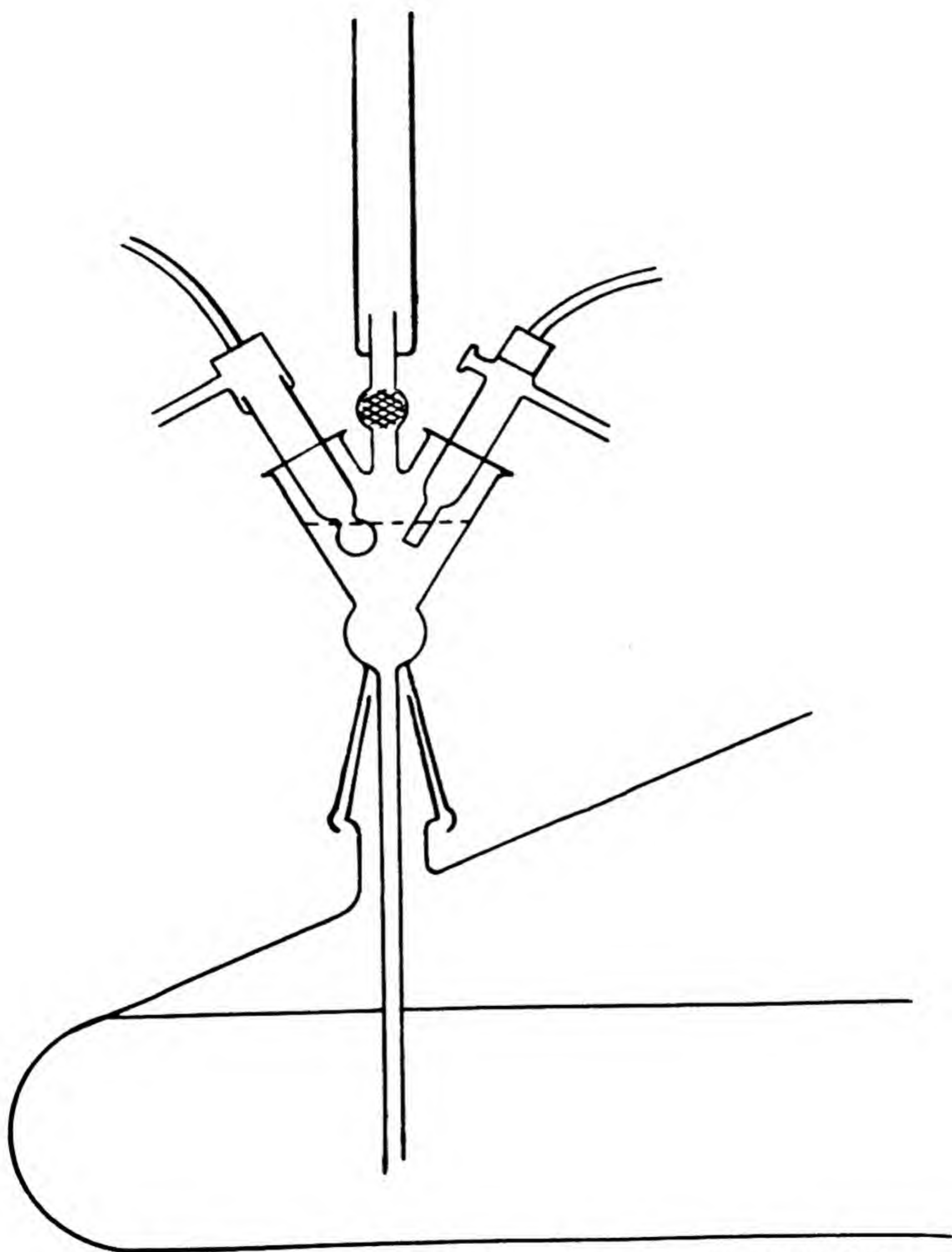


FIG. 19.2—Micro-pH electrode attachment for continuous culture flask.

ments when three inches long. We are indebted to the Division of Plant Nutrition for the culture of barley seedlings used in these experiments.

Preparation of $\text{Na}_2\text{C}^{14}\text{O}_3$.——Barium carbonate containing approximately 4 per cent C^{14} was converted to CO_2 by adding concentrated sulfuric acid from a by-passed dropping funnel to the dry powder *in vacuo*. The evolved gas was collected in a glass spiral trap of 20–60 ml. volume, immersed in liquid nitrogen through which the CO_2 generating apparatus was connected to the high-vacuum line. At pressures of 10^{-4} mm. Hg the quantitative transfer of CO_2 is practically instantaneous. The CO_2 generating assembly was then removed from the spiral trap and replaced by a 10 ml. volumetric flask containing 1–2 per cent excess carbonate-free NaOH, dissolved in a few milliliters of water. The alkali solution was frozen in liquid nitrogen and the flask evacuated, whereupon the CO_2 contained in the spiral (a by-passed spiral is most convenient) was transferred to the flask. After melting, the alkali solution was diluted with water to the given volume. It was found convenient to prepare one to two millicuries of sodium carbonate per 10 ml. solution. For determination of dark fixation rates, the stock solution was diluted 1:25.

Determination of radioactivity.——In all chemical work radioactivity was determined using large-diameter (65 mm.), helium-filled, atmospheric pressure Scott Geiger-Müller tubes operating at approximately 2,000 volts. The mica window thickness varied from 0.79 to 1.3 mg./cm.² The efficiency of such tubes is 5–7 disintegrations per count. Very weak samples, 0–20 cpm. (counts per minute), were measured using an automatic background-sample alternator developed in this laboratory which served to reduce errors due to background fluctuations (35–45 cpm.). In this way, samples could be compared with background in eight-minute cycles as long as necessary. The use of a windowless methane-filled counter such as the Nucleometer will further simplify the counting of weak samples.

Samples of 11.5 cm.² area were mounted on aluminum and glass (1/32 inch thick) disks described elsewhere (10). Carbonate was usually counted as BaCO_3 and gave reproducible and reliable results. Organic samples were counted on both types of plates, depending on acidity and surface tension properties involved. It has been found that self-absorption corrections are negligible for sample thicknesses less than 0.2 mg./cm.²; they have also been measured for thicker layers. In all quantitative work, sample thicknesses were restricted to 0.2 mg./cm.² for uniformly smooth layers of non-crystalline substances. Samples of crystalline substances must be very thin, since the thickness of the individual

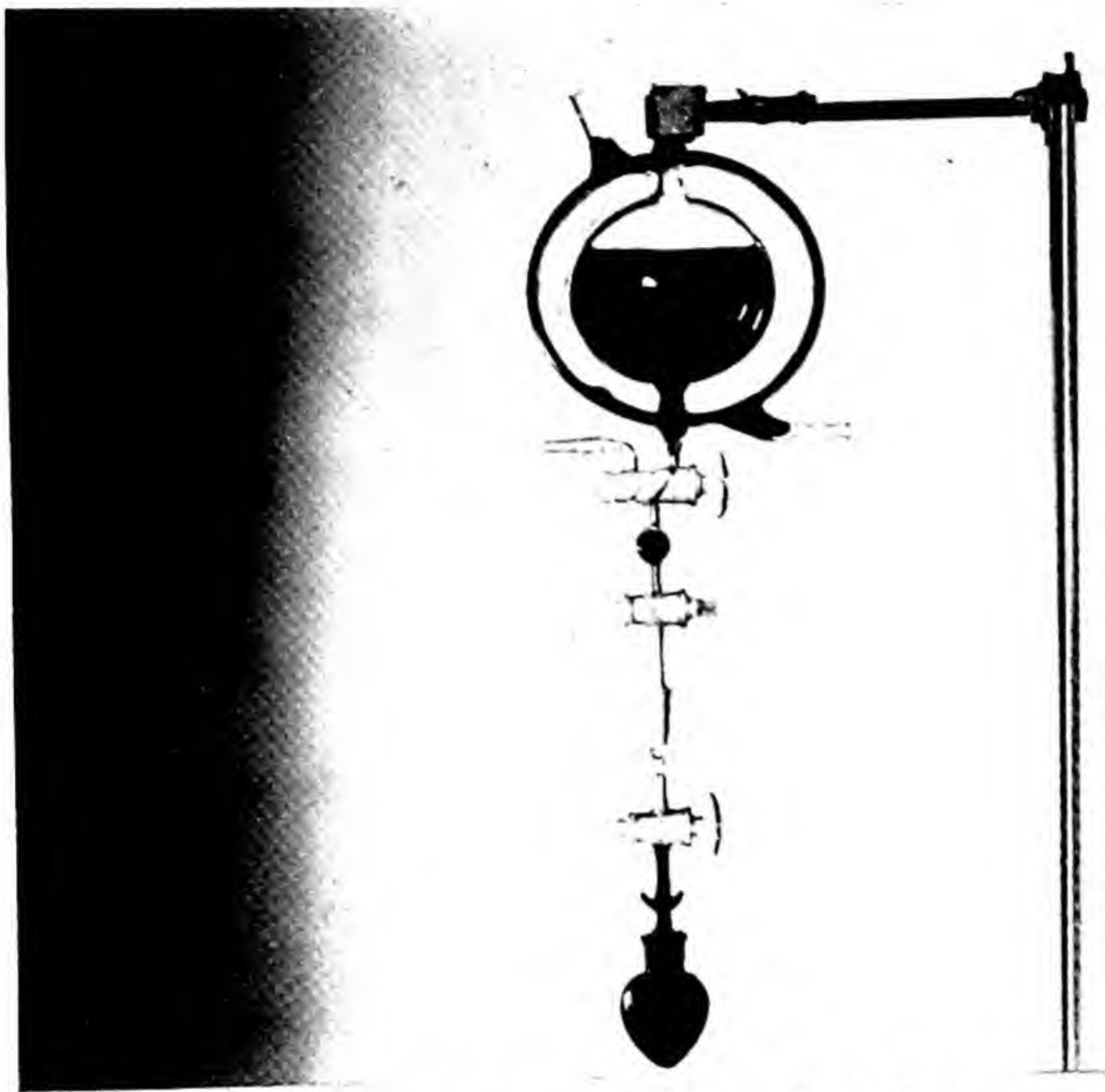


FIG. 19.3—Vessel for preillumination of algal suspensions. Dark fixation is carried out in the small (30 ml.) blackened flask which is detached after receiving an aliquot of algal suspension.

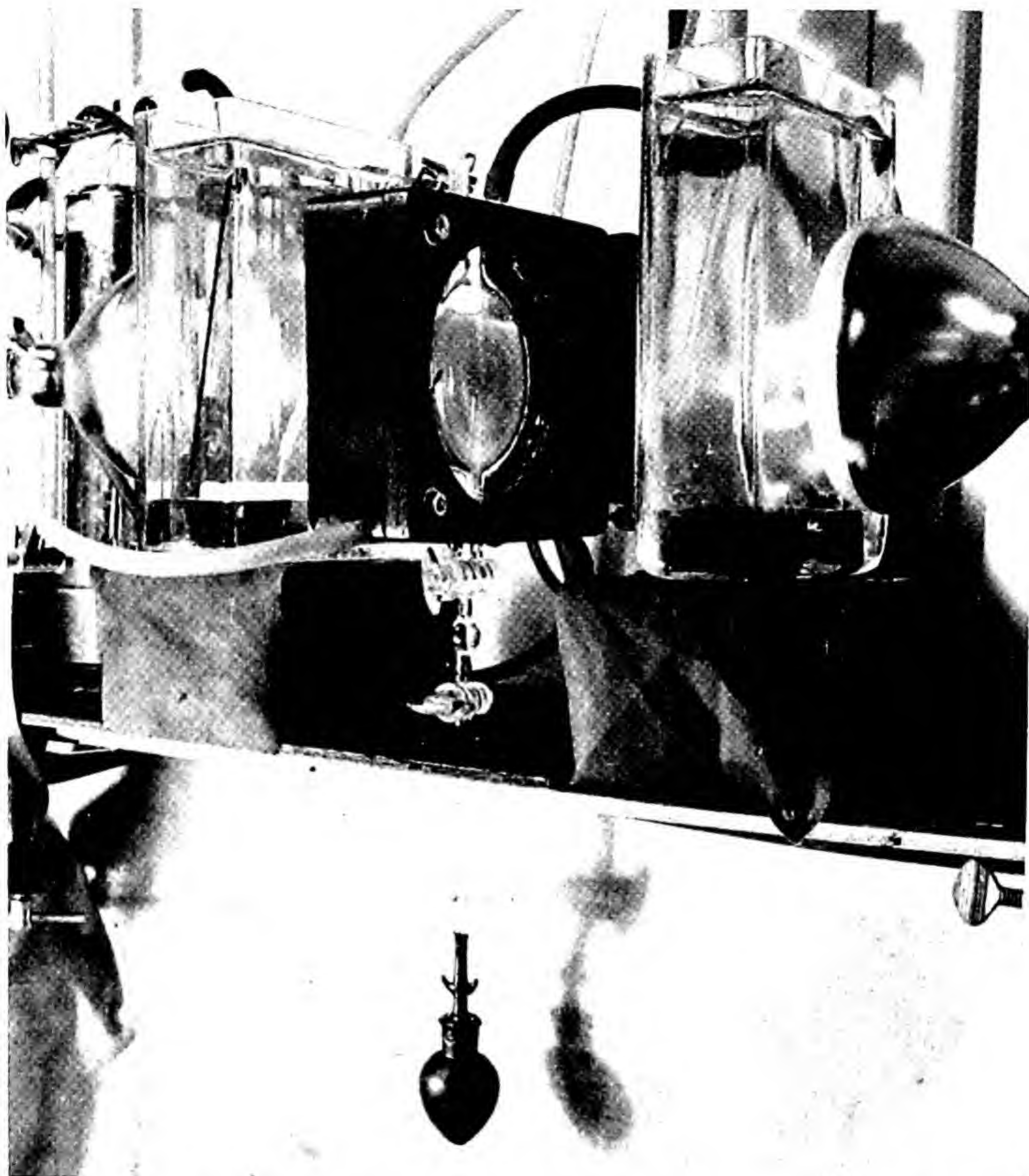


FIG. 19.4—Apparatus for preillumination of algal suspensions. The dark fixation flask is protected from the light beams by being located under a shelf. Helium or 4 per cent CO_2 is admitted to the vessel through the upper tube inserted in the illumination vessel.

crystals will determine the self-absorption of the sample. Such samples give reproducible ($\pm 3\%$) activities, and the results with widely differing materials may be safely and accurately compared, as shown by the activity balances involved in Table 19.2 of this paper.

In degradations and co-crystallizations, specific activities must be accurately determined. Pure crystalline compounds are weighed on the aluminum disks after which the sample (0.50 to 2.50 mg.) is dissolved on the disk in an appropriate solvent and spread on a rotating turntable under a hot air dryer to give a uniform circular film.

Effect of preillumination on dark fixation rates.—The illumination vessel (Fig. 19.3) was a water-jacketed circular vessel 1 cm. thick of 60 ml. volume. Algal suspensions of 1.0–1.5 ml. packed cells in 50 ml. fresh nutrient solution were prepared from fresh, day-old cultures of *Chlorella* and two-day-old *Scenedesmus* cultures. Illumination from both sides of the illumination vessel with 300-watt reflector spot lights using adequate water-cooled glass, infrared absorbing filters was found satisfactory for such cell concentrations (Fig. 19.4). By means of an aliquot bulb equipped with appropriate stopcocks at the bottom of the illumination vessel, a reproducible sample of 1.8 ml. could be withdrawn rapidly and transferred to an evacuated 30 ml. blackened flask containing 0.100 ml. of Na₂C¹⁴O₃ solution (0.8 μ c.). The stopcock attached to the black flask was closed and the sample removed from the illumination vessel. For five-minute dark fixations, shaking was done mechanically while the one-minute fixations were shaken manually. The algae were killed rapidly by adding 0.50 ml. of acetic acid—concentrated hydrochloric acid solution (4:1) to the ground joint above the stopcock and turning the stopcock at the desired killing time. The algal aliquot had been flushed into the black flask with less than one atmosphere of nitrogen pressure, leaving sufficient vacuum to accommodate the acetic acid solution. The fixed non-volatile radioactivity was determined on samples prepared by evaporating 200 μ l. of the suspension on glass disks.

Curve A, Figure 19.5, was obtained by preparing a series of samples from *Scenedesmus* suspension which had been in the dark in 4 per cent CO₂ in nitrogen for one hour, followed by rapid flushing with helium for 20 minutes. The samples were killed after appropriate times of shaking with the labeled carbonate. Curve B was obtained by taking a series of preilluminated samples from the same *Scenedesmus* suspension after illumination (constant helium flushing) for 10 minutes. The initial slope corresponds to a dark fixation rate of 0.1 mm.³CO₂/mm.³ cells/min. at a CO₂ partial pressure of 0.18 mm. (11).

Effect of preillumination upon CO₂-reducing power. — Chloroella and Scenedesmus cultures used for these experiments were those described above. Fresh cells were rapidly centrifuged and resuspended in fresh nutrient and placed in the illumination vessel as with dark fixation rate experiments.

In order to obtain a reliable starting point for the experiments the illumination vessel was darkened and 4 per cent CO₂ in nitrogen

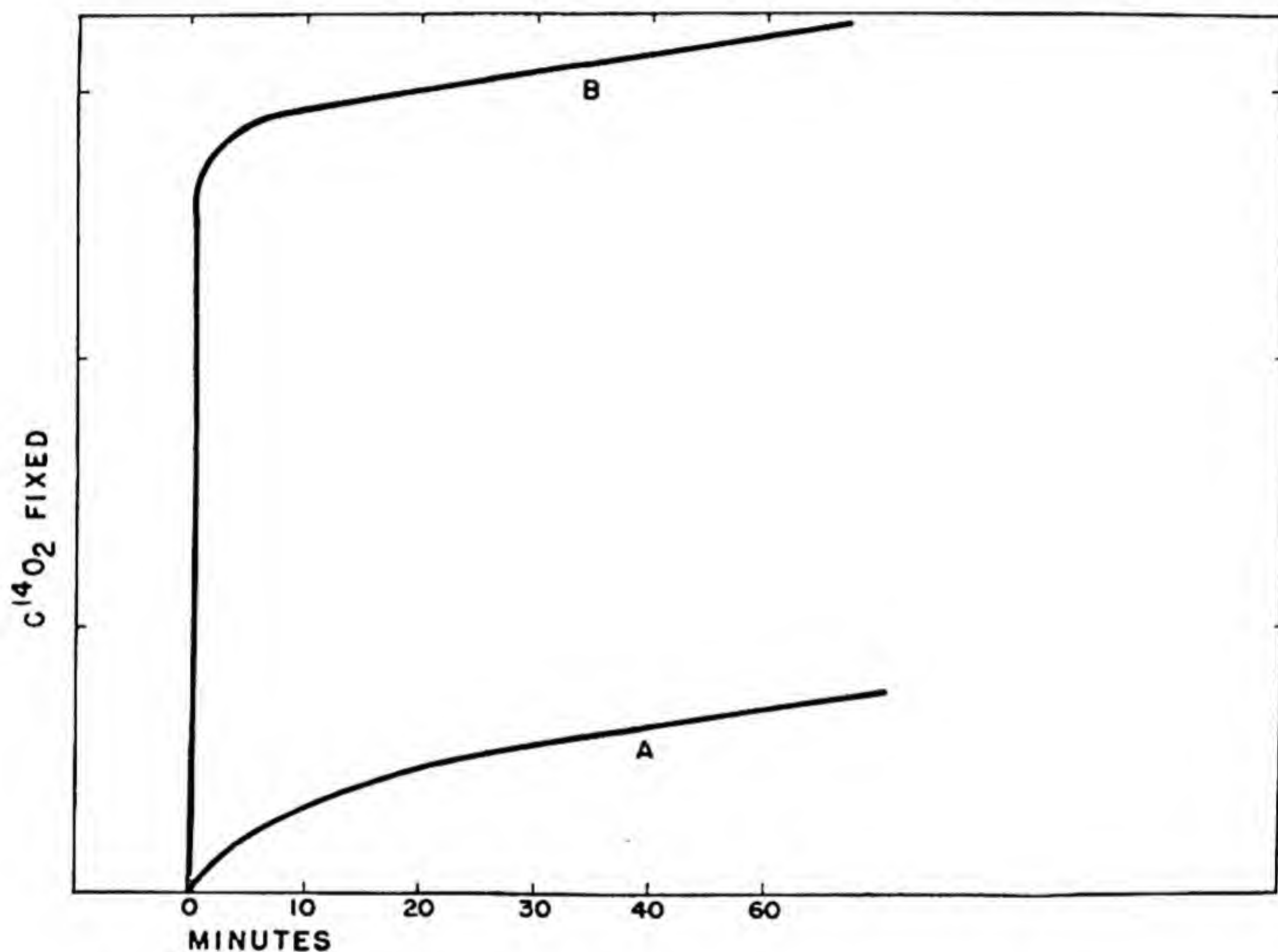


FIG. 19.5—Rate of dark fixation (Scenedesmus). Curve A represents dark CO₂ fixation by one-day-old Scenedesmus cultures after being in darkness one hour in the presence of 4 per cent CO₂ in nitrogen. Curve B represents the dark fixation of CO₂ by the same cells immediately after 10 minutes preillumination.

was bubbled through the cell suspension for one hour, after which it was removed with a vigorous helium stream for 20 minutes. To a large number of black 30 ml. flasks equipped with stopcocks and ground joints was added 100 μ l. (0.82 μ c.) of the diluted Na₂C¹⁴O₃ solution. An aliquot (1.68 ml.) of the algal suspension was added to each of these flasks at suitable times during the experiment. The operation of filling the aliquot bulb and flushing the sample into the black flask with 0.8 atm. of nitrogen pressure required less than

two seconds, during which time the sample was in the aliquot bulb. Several dark samples were always taken to ascertain that the CO₂-fixing power of the algae was constant. These results give the initial straight line in Figures 19.6 and 19.7.

Two 300-watt reflector spotlights were then turned on the illumination vessel, using appropriate water-cooled glass, infrared absorbing filters. Circulation of tap water through the water jacket of the

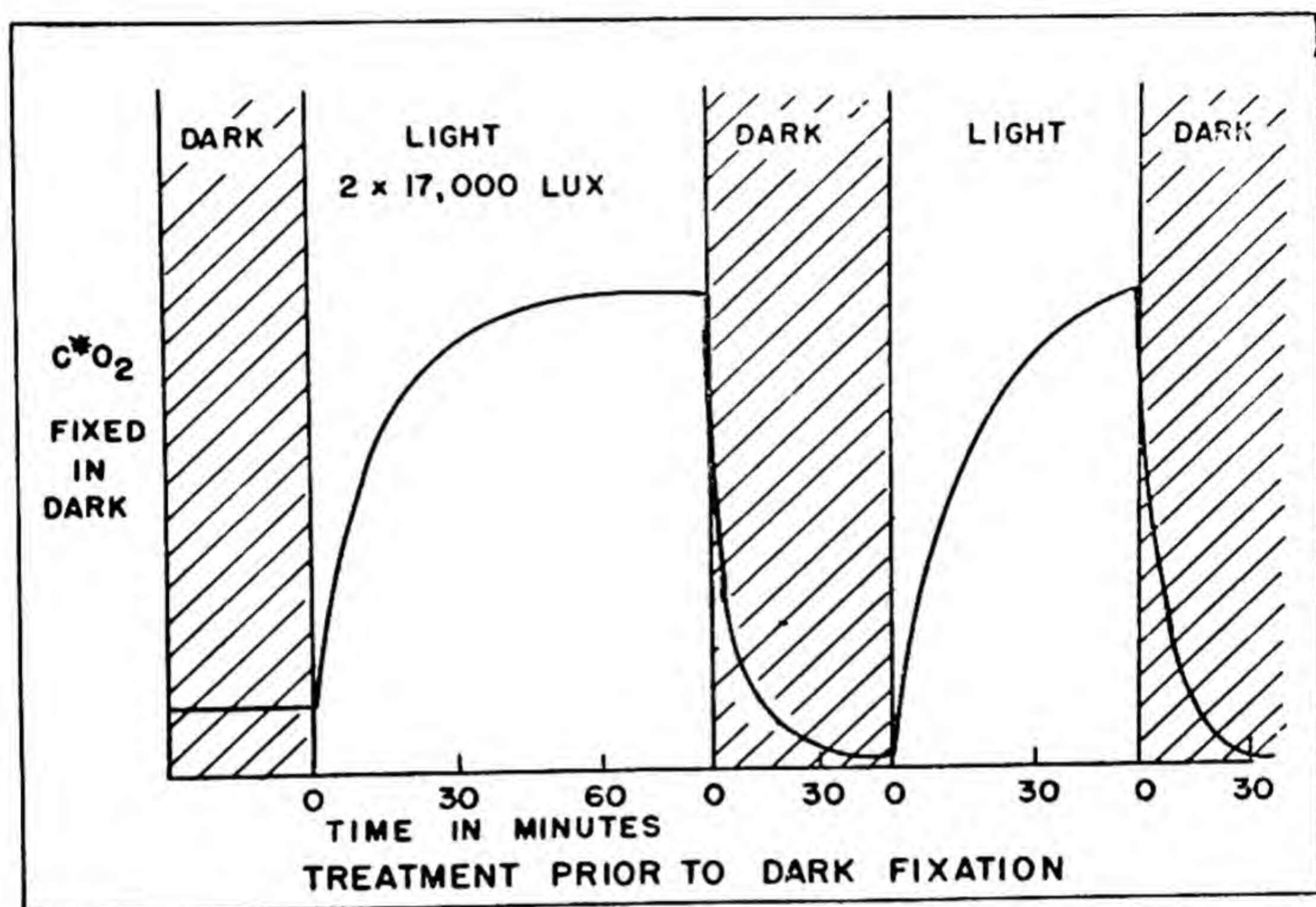


FIG. 19.6—Effect of preillumination on dark C¹⁴O₂ fixation by Chlorella. The curve represents C¹⁴O₂ fixed by equal aliquots of algae during 5 minutes in the dark as a function of the pretreatments described by the abscissa.

illumination vessel maintained the temperature in the algae suspension at 20°C. The curve in Figure 19.6 shows the results with Chlorella in which the aliquot of algae was shaken for 5.0 minutes in the dark with C¹⁴O₂. The curves in Figures 19.5 and 19.7 were obtained with dark C¹⁴O₂-fixation times of one minute.

Experiments in which CO₂-free air was used instead of helium during preillumination gave similar curves. The rate of growth of reducing power was unaffected, but the maximum attained and its stability with time appeared to be diminished appreciably.

Effect of replacing nitrate in the nutrient by ammonium ion on preillumination curves.—Chlorella cells grown in nutrient containing nitrate ion as the source of nitrogen were centrifuged

and washed with a nutrient solution containing only ammonium ion (pH 4.5) as a nitrogen source. The cells were resuspended in this nutrient and their ability to store "CO₂-reducing power" was measured as described above. The experiment was performed in a different manner in that the algae were preilluminated 30 minutes until a maximum was reached. The lights were turned off and the decay rate checked. The "growth" rate following this

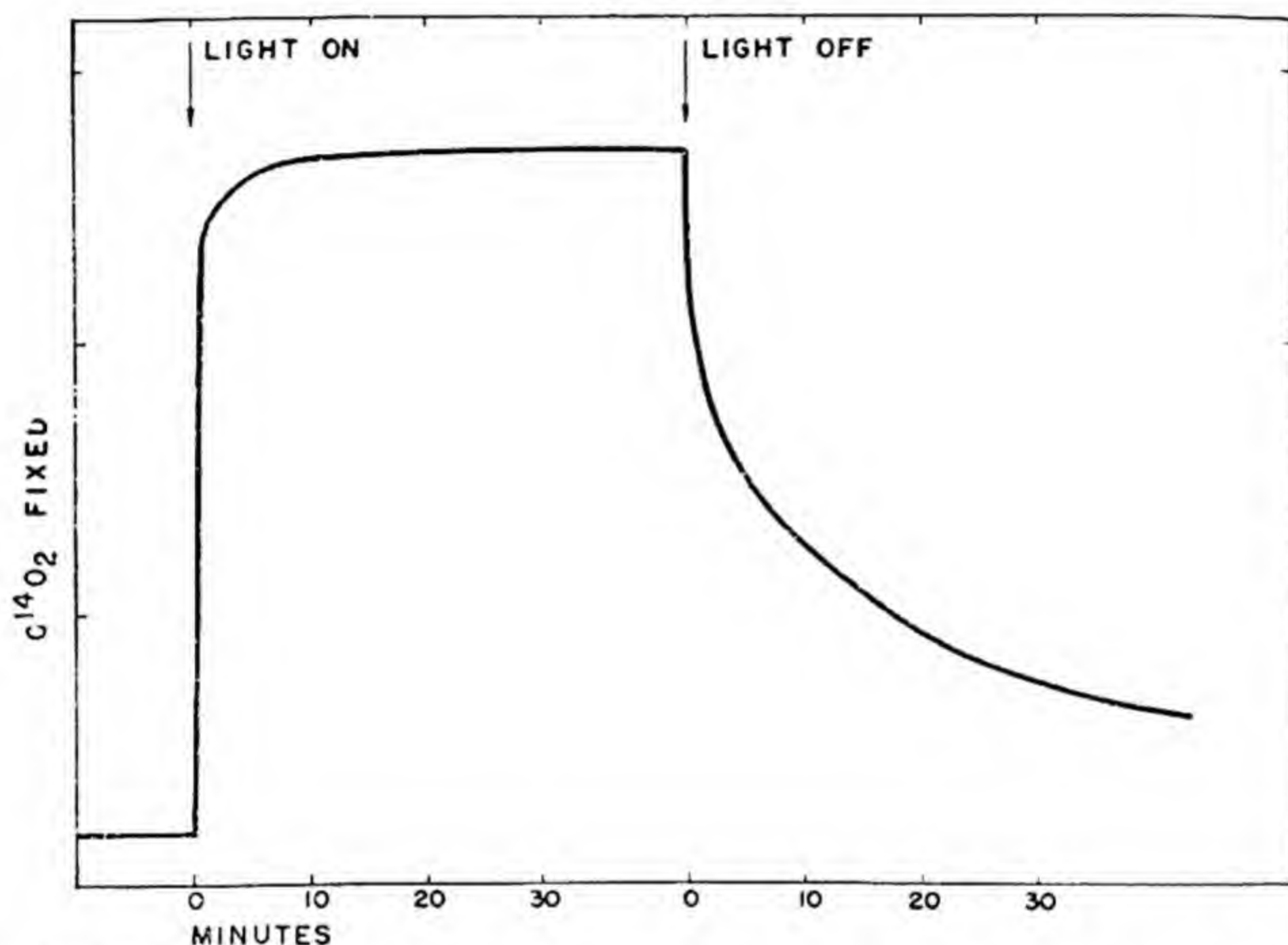


Fig. 19.7—Effect of preillumination upon one-minute dark fixation (*Scenedesmus*).

decay was very similar to that shown in Figure 19.6 for normal, [NO₃⁻]-grown *Chlorella*.

As a check on the validity of this conclusion, a *Chlorella* culture was re-inoculated three times in NH₄⁺ medium and shown to give a "growth" curve identical with that for normal *Chlorella* and for cells merely suspended in (NH₄⁺) medium.

Photosynthetic C¹⁴O₂ fixation by algae.—The illumination arrangement described for preilluminating algal suspensions was adapted to illuminate a 130 ml. circular vessel with flat sides 20 mm. apart. One milliliter of packed cells suspended in 100 ml. of nutrient solution were allowed to carry on photosynthesis with 4 per cent CO₂ in air of the vessel for one hour. The gas was then changed to air for 5 minutes, after which the inlet tube was rapidly removed

from the vessel, and 500 λ (0.10 mc.) of Na₂C¹⁴O₃ stock solution was rapidly injected into the solution. The vessel was stoppered and shaken vigorously in the light beams for the required time. Simultaneously with cessation of illumination, 20 ml. of acetic acid-hydrochloric acid solution was rapidly injected into the body of the solution. After standing 10 minutes in the dark, the products were isolated as described for dark C¹⁴O₂ fixations. In an experiment with similar cells killed by rapidly pouring the solution into boiling ethanol, the activity fixed was the same.

Isolation of radioactive products from algae.——The isolation process described below was developed to separate CO₂-fixation products of dark fixations and short photosynthetic fixations into groups of chemically similar compounds which could be further separated by more specialized procedures. Any fractions discarded or not mentioned were not found to possess significant radioactivity.

The algae suspension, having been rapidly killed by an addition of a fifth of its volume of acetic acid-hydrochloric acid solution, was allowed to stand for 10 minutes during which unused C¹⁴O₂ was recovered by aspiration through sodium hydroxide. The suspension was filtered with Celite into a graduated cylinder. In the case of dark fixations and short photosynthetic fixations, no radioactive products remained insoluble. After determining the activity of a suitably sized aliquot (10–500 μ l.), the solution was subjected to a rapid, continuous ether extraction for 15 hours. The ether extract was evaporated to dryness at reduced pressure and taken up in glacial acetic acid for determination of radioactivity. The aqueous phase was evaporated to dryness *in vacuo* below 20°C., re-dissolved in 20 ml. H₂O for determination of activity, and successively passed through 25 cm. columns containing 25 ml. of Duolite C-3 cation exchange resin and Duolite A-3 anion resin. The effluent sugar solution was rendered acid to phenolphthalein, if necessary, by addition of a drop of dilute hydrochloric acid. The resins were washed with 250 ml. of water, and the total effluente was evaporated at reduced pressure to a convenient small volume for determination of radioactivity (Fraction IV).

Fraction II was obtained upon elution of the cation exchange resin with 100 ml. 7 per cent HCl during one hour. Separation of alanine from this fraction is described below. Further identification of the amino acids synthesized will be described in future publications (12).

Elution of the anion exchange resin was first done with 100 ml. of 1.5 N ammonium hydroxide during one hour or more, after

which the column was washed with 250 ml. of water. Evaporation at reduced pressure to a convenient volume gave Fraction IIA. Further elution with ammonia removed no more radioactivity. The resin was then eluted with 30 ml. of 1.5 N NaOH during one hour. The eluate and the succeeding 250 ml. of water wash was directly passed through a 50 cm. cation resin column (Duolite C-3) which removed the NaOH. The effluete (Fraction IIIB) was evaporated to a convenient volume for determination of radioactivity.

Photosynthesis by barley seedlings.—Gaseous $C^{14}O_2$ was fed to the leaves in the apparatus described previously (13). The leaves were stored in liquid nitrogen after the experiments until extraction. In experiment III, the products were separated by ether and 80 per cent ethanol extractions of lyophilized leaves. In experiments IV and V, the leaves were subjected to repeated extractions with aqueous acetic acid solution. The combined extracts were evaporated to dryness or to a convenient volume and subjected to continuous ether extraction. Succeeding fractionations were similar to those used with algal extracts. The results of these experiments are tabulated in Table 19.3.

IDENTIFICATION OF RADIOACTIVE PRODUCTS

Fractionation of ether-soluble carboxylic acids (I).—The continuous ether extract was evaporated to dryness *in vacuo* and taken up in 0.2 ml. of *n*-butanol. The solution was diluted with nine volumes of chloroform and the separation carried out according to the method of Isherwood (14). For the separation of malic and succinic acid, 5 mg. amounts of each were added as carriers to produce the detectable indicator color as each emerged from the column. The activities so separated were diluted with added carrier for degradation experiments (15).

Isolation of radioactive succinic acid from Chlorella. — Chlorella cultures 10–14 days old were harvested in a refrigerated Sharples super-centrifuge and resuspended in distilled water. To a CO_2 -free suspension of 469 g. of packed cells in one liter of water was added 100 μ c. of $C^{14}O_2$, the mixture shaken vigorously for thirty minutes in the dark after which 100 ml. of acetic-hydrochloric acid solution was added to kill the cells rapidly. The radioactive products were separated from the cells by filtration and subjected to an ether extraction at pH 6.0 which removed very little radioactivity. Continuous ether extraction at pH 1.0 removed 80 per cent of the activity (400,000 cpm.). Evaporation of the ether extract gave 50 mg. of crystalline succinic acid containing a small amount of radioactive fumaric acid. The radioactive product was identified

as succinic acid by determination of equivalent weight, melting point, distribution coefficient between water at pH 1.0 and ether and between water and ethyl acetate, dependence of distribution coefficient on pH of aqueous phase, titration curve, molecular weight, elemental analysis, and by X-ray powder diffraction pattern.

Alanine determination.——To a suitable aliquot of the cation eluate was added 100 mg. of dl-alanine and excess 3 M KHCO₃ solution. N-Benzoyl alanine was prepared in the usual manner by stirring with excess benzoyl chloride. After removing the benzoic acid with ligroin, the product was extracted with chloroform and crystallized from acetic acid-petroleum ether solution (yield, 100–200 mg.). The specific activity of the product was usually constant after one crystallization. The alanine activity of the original aliquot was then calculated from the theoretical yield and the specific activity.

Qualitative identification of radioactive amino acids.——The identification of amino acid constituents of plants as well as identification of radioactive amino acids has been performed using the filter paper chromatography, radioautograph technique (16). The results of these experiments will be reported in future publications (12).

Identification of phosphoglyceric acid.——To a 200 μ l. aliquot of the fraction IIIB (30-sec. photosynthetic *Scenedesmus*) containing 9,250 cpm. of C¹⁴, was added 5.4 mg. of barium 3-phosphoglycerate. The specific activity of the crystalline salt after two recrystallizations from water was found to be 1,650 cpm./mg. for a 0.40 mg. sample. A third recrystallization gave a specific activity of 1,600 cpm./mg. for a 0.29 mg. sample, while a fourth recrystallization was 1,600 cpm./mg. for a 0.49 mg. sample. This assay indicates that 94 per cent of IILA is 3-phosphoglyceric acid but does not represent unequivocal proof since similar compounds may “carry” the radioactivity as well.

Hydrolysis of phosphoglyceric acid was carried out in the following typical manner. To a 141,000 cpm. aliquot of IIIB (from preilluminated *Scenedesmus*) in 10 ml. of 1.0 N HCl was added 3 mg. of barium 3-phosphoglycerate and 15 mg. of sucrose. The solution was heated at 100° in an evacuated sealed tube for 8 days. The hydrolysate was evaporated to dryness *in vacuo*, taken up in water, and was found to have 115,000 cpm. It was adsorbed on a 25 ml. Duolite A-3 column and thoroughly washed with water. Upon elution with 200 ml. of 1.5 N ammonium hydroxide, 82,000 cpm. was found in the evaporated eluate. In separate experiments

it was found that 3-phosphoglyceric acid is not eluted from Duolite A-3 by ammonia while glyceric acid is readily elutable with ammonia.

p-Bromophenacylglycerate was prepared from 112 mg. (0.67 m. mols) of glyceric acid syrup together with the above 82,000 cpm. hydrolysate. It was found advisable to use only 100 mg. (0.36 m. moles) *p*-bromophenacyl bromide in order to obtain a purer product. The specific activity of the crystalline product was found to be 455 ± 50 cpm./mg., which indicates that the hydrolysate activity is all glyceric acid.

Solvent distribution constants for the hydrolysate activity were compared with those of authentic glyceric acid and found to be identical within the errors involved. Distribution of *p*-bromophenacylglycerate (from fraction IIIB of 30-sec. photosynthetic *Scenedesmus*) between phases in a mixture of 0.50 ml. toluene, 0.20 ml. acetic acid, and 0.07 ml. water was carried out using 5.69 mg. (310 cpm./mg.) of the ester. The specific activity of an aliquot of the lower phase was 275 cpm./mg. for a 0.030 ml. (0.90 mg.) aliquot. The organic phase had a specific activity of 308 cpm./mg. The apparent discrepancy between the radioactive ester and the carrier ester was clarified when it was found that 12 per cent or more of the product from the lower phase was water, which caused the low original activity. Evaporation of toluene in preparing a plate of the organic phase had thoroughly dried the sample. Elemental analysis indicated 2-3 moles of water in undried samples of the ester.

Identification tests on compounds appearing in fraction IIIA.—Since carrier triose phosphates are not presently available to us, an unequivocal identification of triose phosphate is not possible. The following chemical properties of the radioactive compounds in ammonia-eluates (IIIA) have been observed.

The ammonia-eluate was evaporated at reduced pressure and readsorbed on Duolite A-3 resin. A small fraction (8-15%) of the activity was found in the effluat and may be hexoses, trioses, or pyruvaldehyde derived from materials in IIIA. Ammonia re-elution removed only 15-20 per cent of the adsorbed radioactivity. The major fraction of the activity was found in the NaOH eluate (after ammonia elution) and was identified as phosphoglyceric acid.

In a separate thirty-second photosynthetic experiment with *Scenedesmus*, tests were performed to identify triose phosphate. Since fraction IIIA was readily converted to a different substance, phosphoglyceric acid, after the first elution from the resin, it was

necessary to use the whole algal extract for identification experiments. To an aliquot was added HCl to make the solution 1.0 N. Carrier dihydroxyacetone was added, and the solution was steam distilled. Pyruvaldehyde dinitrophenylosazone was prepared from the distillate. Recrystallization of the product reduced the specific activity considerably to a specific activity corresponding to 10 per cent of that expected if IIIA were triose phosphate. This did not change upon successive recrystallization. Acetaldehyde dinitrophenylhydrazones were isolated from the steam distillate using carrier. This may be a breakdown product of pyruvaldehyde or phospho-enol pyruvic acid. A small amount of acetic acid was identified in a similar way but no evidence for formic acid or formaldehyde was found.

Identification of neutral substances.——Fraction IV, the efflu-ate from the exchange resins, was evaporated to a small volume, and aliquots were co-crystallized with glucose and fructose to constant specific activities. Although glucose and fructose may not have approximately equal activities as indicated by this method, incomplete information obtained using paper chromatography indicates the formation of both hexoses in dark fixation as well as in short photosynthetic experiments. Glucose phenylosazone was prepared using carrier glucose and fructose from sugars synthesized by photosynthesizing barley seedlings. The total activity in fructose and glucose calculated from the specific activity of the purified osazone was determined and compared with activities in each of the sugars as determined by co-crystallization. Since the sum of the apparent activities in glucose and fructose was 120 per cent of the activity in glucose phenylosazone, it is obvious that one or both of the radioactive sugars had co-crystallized to a certain extent with the other carrier. For degradation experiments glucose was co-crystallized with suitable amounts of radioactive sugars and gave good yields of radioactive lactic acid with *Lactobacillus casei*.

DEGRADATION OF PRODUCTS

Succinic acid.——Samples of labeled succinic acid obtained from separations with the silica gel columns were diluted with carrier and subjected to a Curtius degradation procedure (15). The carboxyl activity is separated as barium carbonate while the methylene carbon atoms are isolated as the hydrochloride of ethylene diamine from the Curtius reaction.

Malic acid.——Samples of malic acid obtained from the silica gel columns were oxidized with chromic acid (15) to give CO₂ from the carboxyl groups and acetic acid from the α - and β -carbon atoms. The CO₂ was counted as barium carbonate and the acetic acid as barium acetate.

Alanine.——N-Benzoyl alanine, of known specific activity, was hydrolyzed overnight in refluxing 48 per cent hydrobromic acid. The solution was evaporated to dryness and the residue washed with ether. The alanine was taken up in water and its activity checked.

The decarboxylation of aliquots containing 10–15 mg. of radioactive alanine with ninhydrin was performed using the evacuated U-tube method of Van Slyke. Acetaldehyde activity was determined by dissolving the theoretical amount of 2,4-dinitrophenylhydrazine in glacial acetic acid into which the products could be distilled. The acetaldehyde-2,4-dinitrophenylhydrazone was recrystallized to constant specific activity from which total activity in the α - and β -carbon atoms of alanine may be calculated. In a separate experiment the acetaldehyde was oxidized to iodoform, the specific activity of which gives the β -carbon activity alone. For determination of carboxyl group-activity it was found necessary to decarboxylate an alanine aliquot in an evacuated small flask equipped with a large bore stopcock through which the CO_2 could later be distilled into dilute carbonate-free sodium hydroxide. The usual procedure which involves heating the alkali in the receiver causes excessive polymerization of acetaldehyde and contaminates the barium carbonate obtained.

Aspartic acid.——Aspartic acid, purified by co-crystallization of carrier with an aliquot of the cation exchange resin eluate, was subjected to the same oxidation procedure as was malic acid.

Glucose.——The degradation of hexose samples was performed using *Lactobacillus casei* according to the method of Aronoff, Barker, and Calvin (17). The validity of this method was confirmed by degradation of synthetic isotopic acetic and lactic acids (18).

RESULTS

The results obtained by the foregoing methods on several preparations of algae and barley are given in Tables 19.1–19.4.

Radioautographs of paper chromatograms of amino acid Fraction II and of the total cell extract for photosynthesizing *Scenedesmus* (30-sec.) demonstrated the predominance of alanine and aspartic acid among the radioactive amino acids as well as the total absence of any detectable glutamic acid. Similar examination of the amino acid fractions of preilluminated algae gave a similar distribution of radioactivity. With preilluminated *Chlorella* the predominant amino acid is alanine while aspartic acid is predominant in preilluminated *Scenedesmus*.

From an examination of Table 19.2 it would appear that the most pronounced difference between preilluminated algae and the photosynthesizing algae (30-sec.) is the large rise in the fraction of radioactivity found in Fraction IIIA.

DISCUSSION

The deductive arguments leading to the following proposed scheme of the path of carbon in photosynthesis have already been

TABLE 19.1
DARK CO₂-FIXATION PRODUCTS OF CHLORELLA^a

Dark Time—Five Minutes ^b					
Preillumination ^c		None	5 minutes	60 minutes	120 minutes
I	Total fixed; relative units	1	~10	~10	~10
	Carboxylic acids in ether extract ^d	52%	21%	14%	11%
	Malic acid ^e	16%	11.5%	7.4%
	Succinic acid ^e	5.2%	3.1%	0.5%
II	Amino acids ^f adsorbed on cation resin	31%	41%	64%	74%
III	Anionic substances ^g adsorbed on anion resin	16%	29%	21%
IV	Sugars non-ionized compounds ^h	0.45%	1.0%	0.96%	1.0%

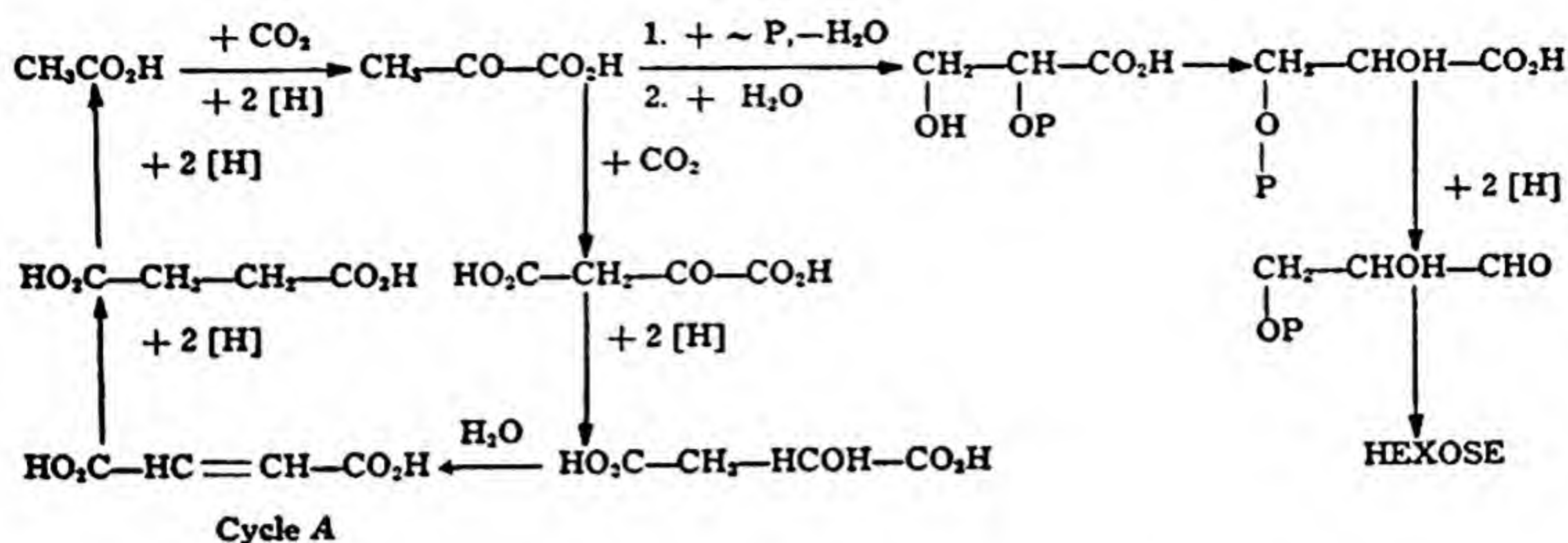
(a) One-day-old cultures of *Chlorella pyrenoidosa*. (b) The cells were killed rapidly by adding 20 per cent by volume of glacial acetic acid-hydrochloric acid (4:1). All radioactive products were in aqueous phase within 5 minutes. Cells removed by filtration. (c) One ml. packed cells per 60 ml. of nutrient solution was illuminated (infrared removed) using 17,000 lux beams from both sides. A rapid stream of helium passed through the suspensions during the experiments. The maximum fixation was not diminished by illumination periods as long as 17 hours. (d) Rapid continuous 15-hour extractions. (e) Separated by partition chromatography on silica gel column. (f) Eluted from Duolite C-3 resin using 2.5 N HCl. (g) Eluted from Duolite A-3 resin using 1.5 N NaOH. (h) Effluat from both exchange resins.

presented (9). Our point of view here will not be to try to demonstrate that it is the only scheme capable of accounting for the results, but rather to show that it can account for presently available experimental data.

TABLE 19.2
DISTRIBUTION OF C¹⁴ IN ALGAE

Alga		Chlorella ^a		Scenedesmus ^b		Chlorella		Scenedesmus	
		60 Minutes		10 Minutes		Photosynthesizing		Photosynthesizing	
		1 Minute Dark		1 Minute Dark		30 Seconds Light ^c		30 Seconds Light ^e	
Total C ¹⁴ fixed, cpm. $\times 10^{-6}$.97	100%	.98	100%	3.1	100%	6.2	100%
I Ether extractable acids		.13	13%	.12	12%	.078	2.5%	.64	10%
II Cationic (amino acids)		.51	53%	.38	39%	.44	14%	.68	11%
III-A Anionic. Ammonia elutable ^d		.023	2.4%	.041	4.2%	1.19	38%	2.75	44%
III-B Anionic. Not ammonia elutable ^e		.30	31%	.41	42%	1.13	36%	1.67	27%
IV Neutral substances (sugars)		.0033	0.3%	.0013	0.1%	.14	4.5%	.29	4.7%

(a) *Chlorella pyrenoidosa*, one-day-old cultures. (b) *Scenedesmus D₃*, two-day-old cultures. (c) Cells rapidly photosynthesizing were given radioactive carbonate and shaken until removed from the light beams and instantly killed. (d) Adsorbed on Duolite A-3 and eluted with 1.5 N ammonium hydroxide. (e) Eluted with NaOH following the previous ammonia elution.



An examination of Cycle A, running in a clockwise direction, shows that its net result is the reduction of two molecules of CO₂ to one molecule of acetic acid. The energy required to accomplish this is obtained in the form of reducing equivalents, designated [H], from the photochemical apparatus involving chlorophyll and other substances. We do not say that all of these reducing equivalents have their immediate origin in the photochemical apparatus, and we cannot say, as yet, which ones do.

It is clear from an examination of the cycle that, if it is running with tracer CO₂, the tracer will appear first in the carboxyl groups of the three- and four-carbon acids, then, in the carboxyl group of the acetic acid and the α-carbon atoms of the three- and four-carbon acids, and, finally, in the methyl group of the acetic acid and of the β-carbon atoms of the pyruvic acid. If the cycle should be stopped (the plant killed) after a relatively short period of operation, the specific activity of the carbon in each of the above named positions will be found to decrease in the order named. This is in accord with the available data shown in Table 19.3.

There are at least four positions at which the products accumulated in this Cycle A may be drained off to produce the normal constituents required by the plant for its life, growth, and storage. Thus, the pyruvic acid may be drained off through glyceric acid and glyceraldehyde to hexose by the direct reversal of the usual glycolytic mechanism. This would produce a hexose in which the specific radioactivity would be highest in the center pair of carbon atoms, decreasing toward each end. The earliest amino acids which are formed, namely alanine (serine) and aspartic acid, probably have their origin in the pyruvic acid and the oxalacetic acid, respectively. Finally, the two-carbon fragment corresponding to acetic acid may undergo β-condensation to acetoacetic acid and form fats.

It should be pointed out that the compounds as given in the above scheme merely represent the nature of the chemical trans-

TABLE 19.3
PHOTOSYNTHETIC PRODUCTS OF BARLEY

	Experiment III ^a	Experiment IV ^b	Experiment V ^b
Age.....	10 days	7 days + 14 days at 5°C.	11 days
Pretreatment.....	15 min. dark	15 min.	52 min. dark
Time photosynthesizing....	75 min.	47 min.	60 min.
Dark respiration time before freezing.....	5 min.	6 hours	0 min.
Total C ¹⁴ fixed (cpm.).....	94 × 10 ⁶ (100%)	22 × 10 ⁶ (100%)	98 × 10 ⁶ (100%)
Insol. in 80% alcohol.....	25%		
Insol. in acid.....		9%	12%
I Ether ext. pH 1.....	1%	3%	3%
II Cation resin eluate....	3.3%	4%	3.4%
III Anion resin eluate....	2.9%	1.2%	2.5%
IV Neutral substances in resin eluate.....	58%	88%	52%

(a) Extraction as in (13). (b) Extraction as of algae.

TABLE 19.4
DEGRADATION OF PHOTOSYNTHETIC PRODUCTS
Figures Represent Percentage of C¹⁴ in the Various Carbon Atoms

Compound Degraded	60 Min. Preillum. Chlorella 30 Min. Dark Fix. C ¹⁴ O ₂	60 Min. Preillum. Chlorella 30 Min. Dark Fix. CH ₃ C ¹⁴ O ₂ H	60 Min. Preillum. Chlorella 5 Min. Dark Fix. C ¹⁴ O ₂	30 Sec. Photo. Scenedesmus	40 Min. Photo. Barley II	75 Min. Photo. Barley III
Succinic acid						
-CH ₂ -.....	2.5					37
-CO ₂ H-.....	97.5					63
Malic acid						
-CH ₂ -CHOH-..			0.86	6.5		
-CO ₂ H.....			99	93.5		
Alanine						
-CO ₂ H.....	89	66	98.1			26 ^a
-CHNH ₂ -.....	10					49
-CH ₃	0.5	34	2			25
Aspartic acid						
-CO ₂ H.....				96		
Residue.....				4		
Glucose						
3,4.....	76			87	61	36
2,5.....	17			6.5	24	37
1,6.....	7			6.5	15	27

(a) The low C¹⁴ content of the carboxyl group is probably due to the low specific activity of C¹⁴O₂ photosynthesized by the plants during the last part of the experiment.

formations, and they are not intended to specify the precise chemical form through which the transformations take place. These are all almost certainly enzymatic transformations. Each of the above listed compounds in the cycle may undergo the whole scheme of transformations without ever becoming free molecules unattached to their corresponding enzymes, or they may be passed from enzyme to enzyme in some specially activated form; for example, the acetic acid may be passed around only as an acetyl group attached, perhaps, to phosphate or in some other special form. Our method of isolation would not allow the detection of any labile form of these intermediates, since all such forms would have had ample opportunity to hydrolyze down to the simple compounds as shown.

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Kinetics of a Photochemical Intermediate in Photosynthesis

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THE LAST thirty years have seen the nearly complete elucidation in terms of intermediates and of enzymatic catalysts of two fundamental metabolic processes—respiration and fermentation. Of the third, photosynthesis, not a single intermediate substance or catalyst appearing or acting in this process on the way from CO_2 to carbohydrates has been identified with certainty. This is true despite the fact that numerous investigators have collected a wealth of information concerning the reaction rates and the photochemical requirements of photosynthesis. Until recently two explanations were advanced to explain this situation, the first being that a reduction of CO_2 is possible only by means of a photochemical process, and that this required a type of reaction differing completely from all other metabolic processes; the other, that as an entirely synthetic process it needed the organization of the intact cell in order to proceed, while breakdown reactions not being coupled with endergonic processes could run their course without the aid of the living structure.

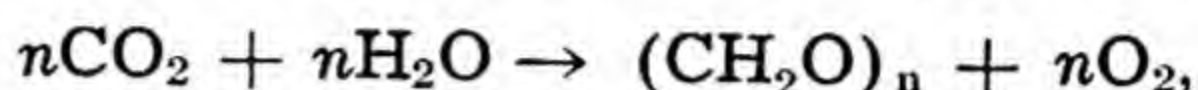
The falsity of the first explanation became evident when it was shown that living cells of all kinds were able to fix and even reduce CO_2 in conjunction with their ordinary dark metabolism. The second explanation was considerably weakened by the discovery that the division of the photosynthetic process into the three component parts (photochemical process, CO_2 reduction, and O_2 evolution) required to explain kinetic data could be demonstrated experimentally. Oxygen evolution may be brought about photochemically

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by illuminated chloroplast preparations without the concomitant reduction of CO_2 . The reduction of CO_2 may be demonstrated as either a photochemical reaction without an accompanying evolution of oxygen or as a dark reaction in which light energy is replaced by an energy-yielding chemical process.

Because the equation for the over-all reaction in photosynthesis,



is the reverse of the over-all equation for carbohydrate respiration, it has been repeatedly suggested that photosynthesis is simply a reversal of respiration and proceeds via the same intermediates. The results of the investigations of the dark fixation of CO_2 made this hypothesis so attractive that the solution of the whole problem seemed to depend only upon a method which would allow for the intermediates produced in the course of photosynthesis to be distinguished from the same intermediates produced by respiration. The carbon isotopes made such distinction possible, and many biochemists expected the very first experiments would prove that the stepwise reversal of respiration was a fact and not a mere hypothesis.

The present work, in which C^{14} has been used to trace the path of carbon in the illuminated cell, has yielded results which indicate that the theory of a simple reversal of respiration is probably wrong, and that it is not wise to ignore (seduced by the apparent simplicity and infallibility of the tracer method) the well established knowledge about photosynthesis derived from previous investigations. It will be shown that the substance in which photochemically incorporated CO_2 first appears in a chemically and thermally stable form has the properties required by the previous theoretical analysis of kinetic data.

In tracing the transformation of labeled carbon during the course of photosynthesis, what kind of compound can we expect to find? According to present knowledge, all that seems necessary to accomplish the purpose of photosynthesis is that the original carbon of CO_2 become a part of a triose-phosphate molecule. From this point, all necessary chemosyntheses can proceed without the aid of light. Therefore, the first compound of carbohydrate nature, or in energetic equilibrium with carbohydrate, appearing in the photosynthetic process we may call a "finished" product. Even this first one, resulting from the last of a sequence of photochemical reactions, is likely to reveal only part of the way along which it originated. We may expect this first product to be an especially reactive metabolite since it is the ultimate precursor of every organic substance within the living cell.

Preceding the first "finished" product and its derivatives will be less reduced intermediates. Any true intermediate of photosynthesis must be a photosensitive compound, a substance still subject to reduction under the influence of the illuminated chlorophyll-protein complex. Among such compounds, the one least reduced should be set apart as the product of fixation of CO_2 , photosensitive, of course, but of chemosynthetic origin. There are compelling theoretical reasons why CO_2 , as such, cannot enter the first of the photochemical reactions. Hence it is the consensus that the first step in the reaction sequence of photosynthesis is a chemosynthesis, or "dark reaction," which fixes CO_2 . By analogy with the recently discovered metabolic fixation reactions, it can be expected that this particular fixation product should easily decompose into CO_2 and the acceptor while being extracted from the plant. Indeed, there is indirect experimental evidence which indicates the reversibility of this first reaction. Thus, in trying to identify the fixation product, one faces the difficulty of instability plus that of having to prove that a dark fixation product obtained from the living plant is truly a precursor of photosynthesis and not simply one of those now known to participate in purely chemosynthetic reactions.

Among the three kinds of substances we may expect to find in the course of photosynthesis—the labeled carboxylic acid formed by CO_2 fixation in the dark, the first "carbohydrate," and the photochemical intermediates—only the last are likely to be peculiarly characteristic of photosynthesis in the sense that they do not appear among the regular intermediates of carbohydrate breakdown. In contrast to the dark fixation product, these intermediates must be quite stable because photosynthesis proceeds with excellent yield at low light intensities even under conditions where the individual chlorophyll molecules in the plant cell have hardly a chance, during the entire course of an experiment, to absorb enough light quanta to bring about the reduction of a molecule of CO_2 . We shall not cite the well known discussions centering around the so-called "photosynthetic unit." Suffice it to say that we need a mechanism whereby the energy of a single light quantum is effectively stored as chemical energy until the next photochemical step can take place.

One way in which the free energy released in respiration is put aside for future use is by the formation of energy-rich phosphate bonds. A particularly good example, showing the interrelation of the fixation of free CO_2 during a chemosynthetic process and the formation of organic phosphates, was found in the metabolism of *Thiobacillus*. This organism oxidizes sulfur and simultaneously produces organic phosphate, probably ATP. The latter is then utilized in connection with the fixation of CO_2 . These observations by Vogler and

Umbreit (1) are now customarily cited in connection with the problem of photosynthesis, for the sulfur bacteria are related in their metabolism to the sulfur purple bacteria, and the latter to the green plants. No analogy would be more welcome. However, the conversion of each light quantum to one energy-rich phosphate bond does not provide the necessary energy, and conversion to more than one phosphate bond requires improbable assumptions (2). Furthermore, no aftereffect of light in any way comparable to the dark fixation observed in *Thiobacillus* has ever been found in green algae. The relation between the fluorescence of chlorophyll and the concentration of CO_2 is further powerful evidence against any hypothesis which involves photochemical formation of a reductant and subsequent dark reduction of CO_2 separate from the chlorophyll-protein surface (cf. Franck, Chap. 16). If we reject then, as we are forced to, the aid of an intermediary, energy-rich phosphate, the necessary stability must reside in the intermediate products of each photochemical step. We have to assume that these are formed immediately and directly in contact with the illuminated chlorophyll complex. The experiments presented below are definitely in favor of this alternative.

The use of carbon isotopes for the study of photosynthesis did not begin with C^{14} which is presently so easily available. In 1939 C^{11} with a halflife of twenty-one minutes was produced with the aid of the cyclotron at Berkeley. Ruben, Kamen, and Hassid (3) were then able to show with photosynthesizing *Chlorella* cells that in illumination periods of thirty minutes or more C^{11} was distributed throughout the cell. They, therefore, shortened their time of exposure to five minutes or less. Under these circumstances the radioactive carbon was found to remain in a water-soluble fraction, not extractable with organic solvents and not identifiable as one of the more common intermediates entering into the cycle of dark reactions in living cells. These authors were handicapped by the short halflife of C^{11} and were thus forced to work rapidly and add artificial carrier material. We would like to emphasize, therefore, that, using natural carrier material and with ample time for careful chemical work, we have been able to confirm the main points of their work. This means, however, that both the early work with C^{11} and our recent experiments with C^{14} cannot be reconciled with the results and conclusions published by Benson and Calvin (4).

ASSIMILATION OF TRACER IN THE LIGHT

The major problem confronting us at the beginning of our investigations of photosynthesis with C^{14} as tracer was how to establish with certainty the position along the path from CO_2 to carbohydrate of the substance which had accumulated tracer carbon

during the period of illumination. Was it the dark fixation product of CO_2 ; was it a photosensitive intermediate; or was it the first "carbohydrate"? The approach we chose was an obvious one. Illuminated suspensions of the green alga, *Scenedesmus obliquus*, were exposed for various periods of time to carbonate enriched with C^{14} , and then killed as rapidly as possible. The algal material was separated into several gross chemical fractions on the basis of solubility, and the radioactivity was determined in each.

A number of separation schemes were tried, including one very similar to that used by Ruben *et al.*, viz., extraction with hot dilute acid. All schemes gave consistent results in that in the shortest experiments the largest percentage of the total radioactivity fixed was always found in the fraction containing compounds soluble in water but insoluble in organic solvents.

In long experiments (one-half to several hours), we found that all fractions became strongly radioactive, indicating a wide distribution of tracer among the diverse cellular constituents. However, in shorter runs (a few minutes down to 30 sec.), while less total tracer was taken up, a larger percentage of the tracer was localized in one fraction. Figure 20.1 illustrates the time course of the distribution of tracer among three arbitrary fractions. Figure 20.2 gives the separation scheme which, in brief experiments, resulted in the concentration in one fraction of the highest percentage of incorporated tracer. This fraction we have labeled *B* and, since the terminology is arbitrary, we shall call the corresponding water-soluble fraction from other separation procedures fraction *B* as well. The curve for percentage of tracer in fraction *B* appears to extrapolate to 100 per cent at zero time. Apparently the tracer first accumulates chiefly in fraction *B* and is converted by subsequent reactions into compounds of other types, many of which are in fractions *A* and *C*. We feel confident that fraction *B* contains at least one substance which occurs fairly early in the chain of photosynthetic events. Therefore, we proposed (a) to attempt to isolate the substance responsible for the radioactivity in this fraction and (b) to investigate the factors which influence its transformation into compounds contained in the other fractions.

CHARACTERISTICS OF A PHOTOCHEMICAL INTERMEDIATE

In order to study the photochemical intermediate (which we shall call "*B*"), shown to be present in fraction *B*, it is important to obtain it as free as possible from later products of photosynthesis. In long experiments components of fraction *B*, other than "*B*," could become tagged by secondary reactions, thus rendering more difficult the identification of "*B*" as distinct from other tagged substances which may be unrelated to photosynthesis. Therefore, the

photosynthesizing cells must be exposed to tracer CO_2 for only a brief interval, for it would be exceedingly fortuitous if fraction B contained nothing but "B", i.e., if it were chemically homogeneous.

In order to obtain sufficient material for chemical manipulation, we cultured *Scenedesmus* autotrophically in a 100-liter tank. In

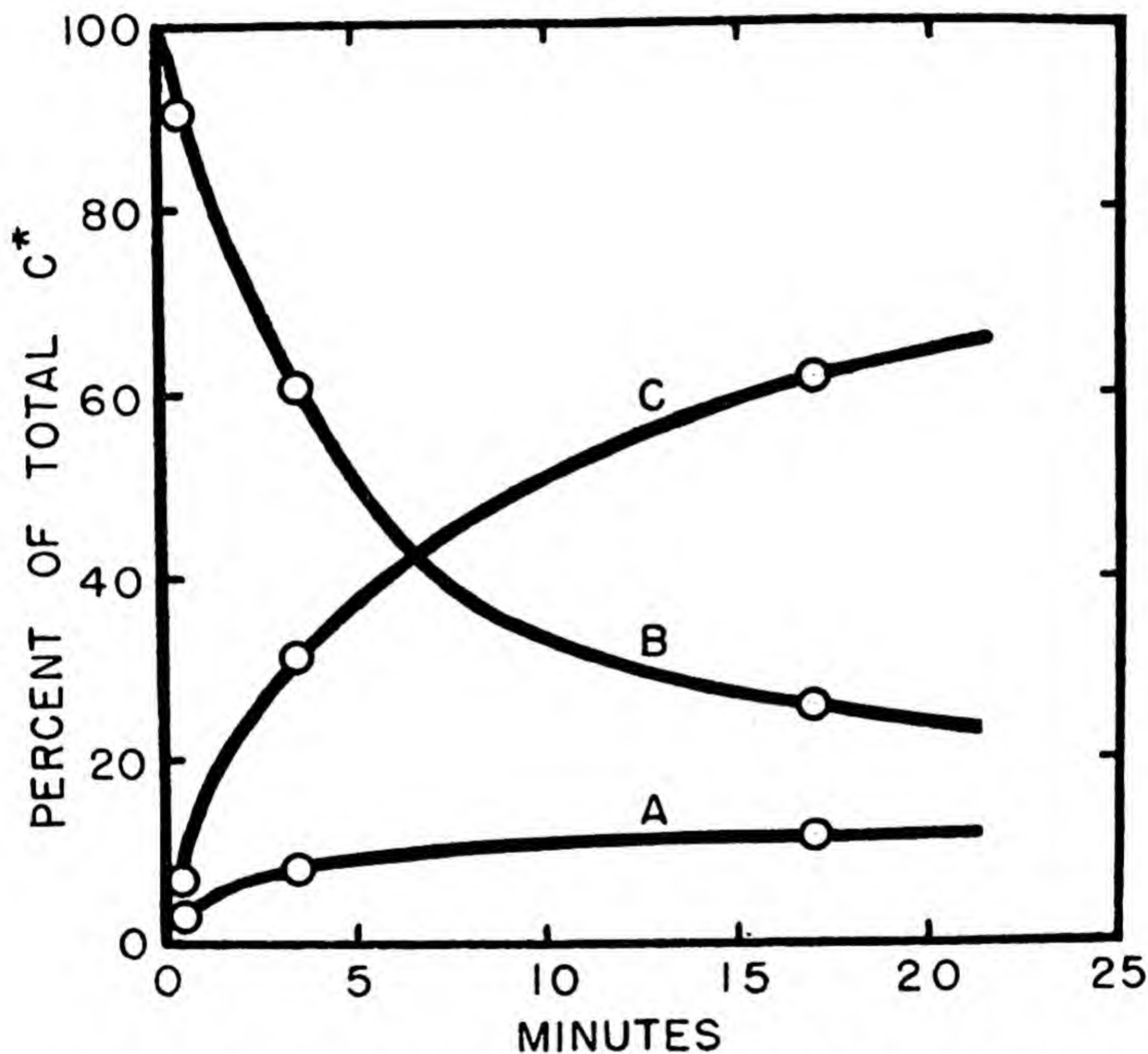


FIG. 20.1—Time course of tracer distribution during photosynthesis. Letters refer to the fractions into which algal components were separated. Each point represents tracer found in a particular fraction as a percentage of total tracer incorporated by the cells.

about three days from the time of inoculation, between 100 and 200 g. of wet algae were harvested. It would be difficult to tag with C^{14}O_2 for a short time and to kill rapidly 100 g. of algae. Therefore, a sample of algae (ca. 0.5% of the crop) was suspended in buffer and illuminated in successive small aliquots. These photosynthesizing cells were exposed to radioactive CO_2 for about thirty to forty seconds and then were killed quickly, either in boiling

water, boiling alcohol, or hot acid as required by the subsequent fractionation procedure. In the case of the scheme shown in Figure 20.2, hot ethanol was the killing agent. The bulk of the algal crop was killed in the same way, and the extracts of the small sample of tagged algae were mixed with those of the much larger

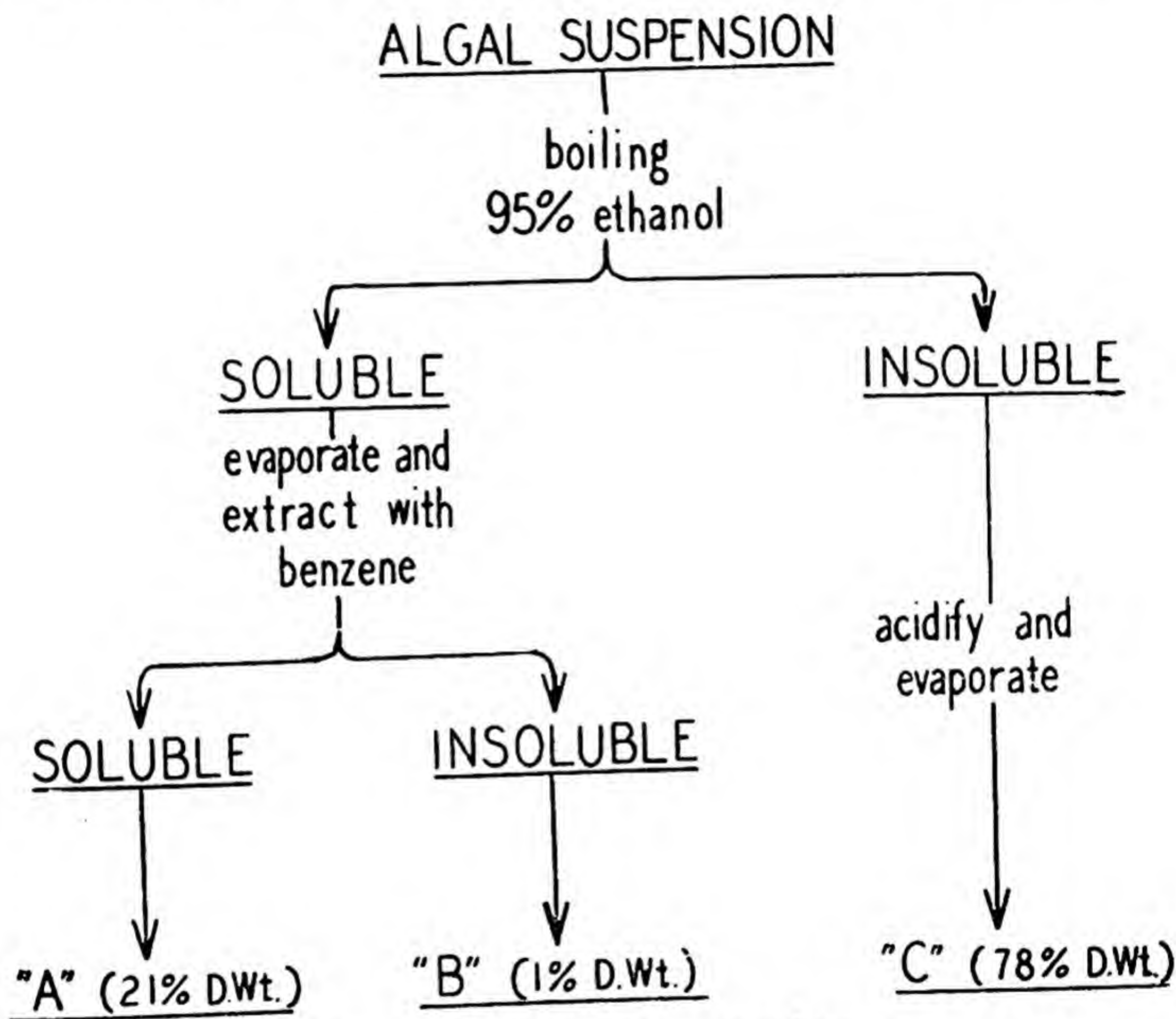


FIG. 20.2—Fractionation procedure.

batch of non-radioactive cells. Chemical investigation has been confined to such material. Attempts at purification of fraction B have not yet led to the isolation of a crystalline product. It has been shown that "B" is not a carbohydrate (or phosphate ester of such a compound), an amino acid, a keto acid, any acid of the metabolic cycle, an aldehyde, ketone, phenol or alcohol, or a polyhydroxy acid (or phosphate ester of such a compound), (cf. Fager, Chap. 21). It should be pointed out that the "light product" studied by Ruben and co-workers (3) in *Chlorella* was also characterized mainly by what it could *not* be.

Even though chemical identification of "B" must await further experimentation, we can draw positive conclusions as to its role

in the reaction sequence of photosynthesis. We have studied the factors which affect the transformation of "B" into compounds present in the other fractions by varying the environmental conditions during the exposure to tracer CO_2 as well as after the tracer carbon had been assimilated. A number of interesting properties of "B" *in vivo* have been established:

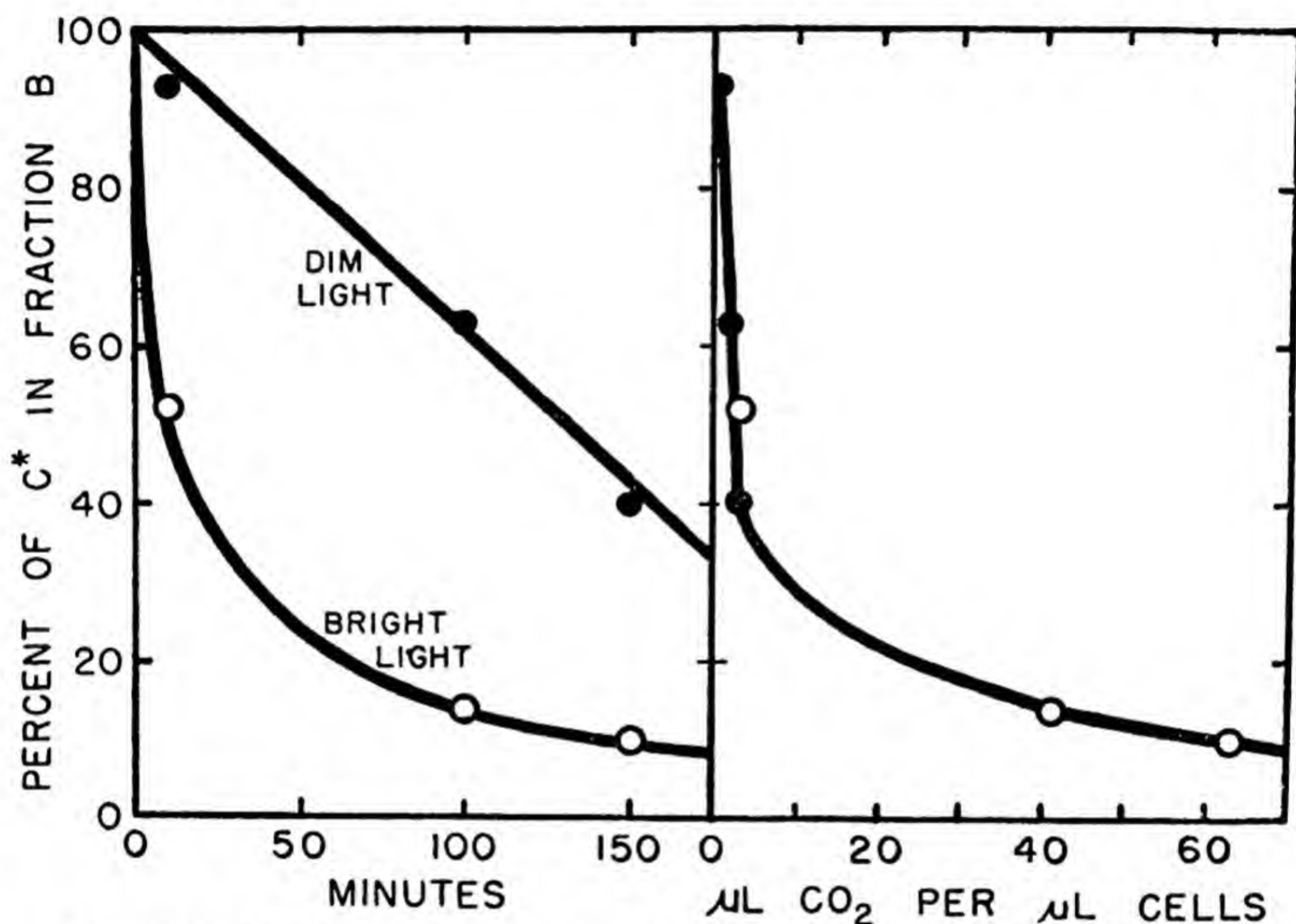


FIG. 20.3—Relation of tracer distribution to time of assimilation and to total photosynthesis. Data for two light intensities are plotted. Graph on left shows that tracer was shifted out of fraction B most rapidly at high light intensity. When abscissa scale of graph on left is multiplied by the respective photosynthetic rates, the graph on the right is obtained. Thus the percentage of assimilated tracer remaining in fraction B is a function of total assimilation.

The rate of transformation of tracer from fraction B into other fractions depends upon light intensity.—The time curves of Figure 20.1, representing percentage of total tracer in the several fractions as a function of time, apply only to one light intensity, i.e., to one particular assimilatory rate. By varying the light intensity, similar time curves representing different rates of photosynthesis were obtained. Each time course of tracer distribution thus constitutes one set of a family of such sets of curves. The data of Figure 20.3 illustrate the difference between time curves for fraction B at a low rate of photosynthesis (just above compensation) and at a high rate (near light saturation). However, when the same data are plotted against total assimilation rather than time,

points for high and low light intensities fall on the same curve, as shown in the right half of Figure 20.3. This result can be explained by various kinetic theories, all of which include the assumption that the transport of tracer carbon from fraction *B* into other fractions is a first order reaction.

Transformation of "B" into compounds of the other fractions does not occur in the dark.——If the shift of tracer from fraction

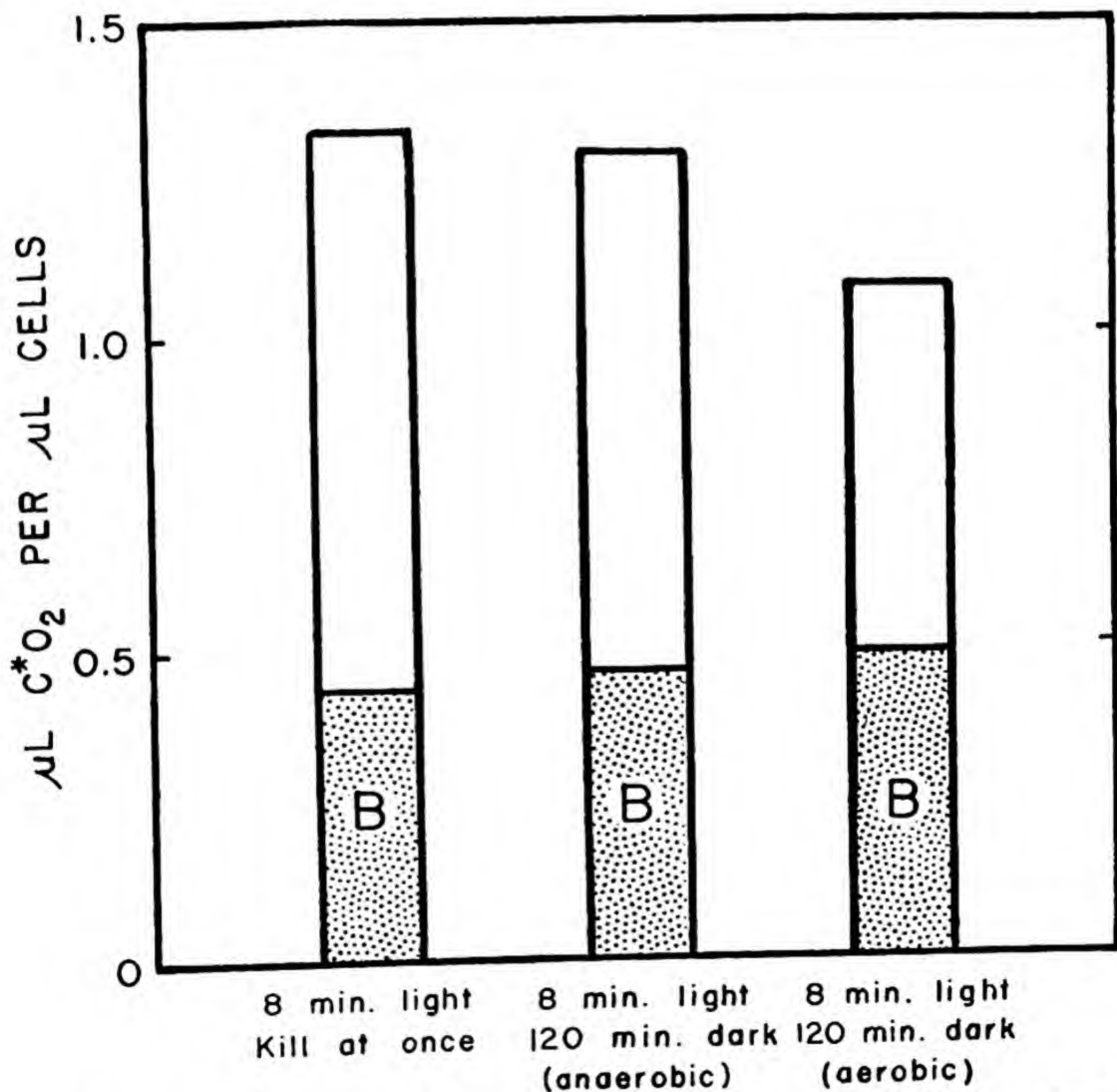


FIG. 20.4—Effect of aerobic and anaerobic conditions on tracer previously incorporated by photosynthesis.

B were a non-photochemical reaction, one should expect it to proceed to equilibrium in a dark interval subsequent to tagging of "B" by photosynthesis. However, the transformation stops abruptly as assimilation stops. Figure 20.4 shows that a two-hour dark period after the initial tagging resulted in no clearly measurable

shift of tracer out of fraction B. This was true for aerobic as well as anaerobic experiments. The only difference was that aerobically the cells as a whole lost some tracer carbon, presumably by respiration. This loss occurred entirely from cell constituents other than "B." Therefore, "B" is metabolically stable in the dark. The most

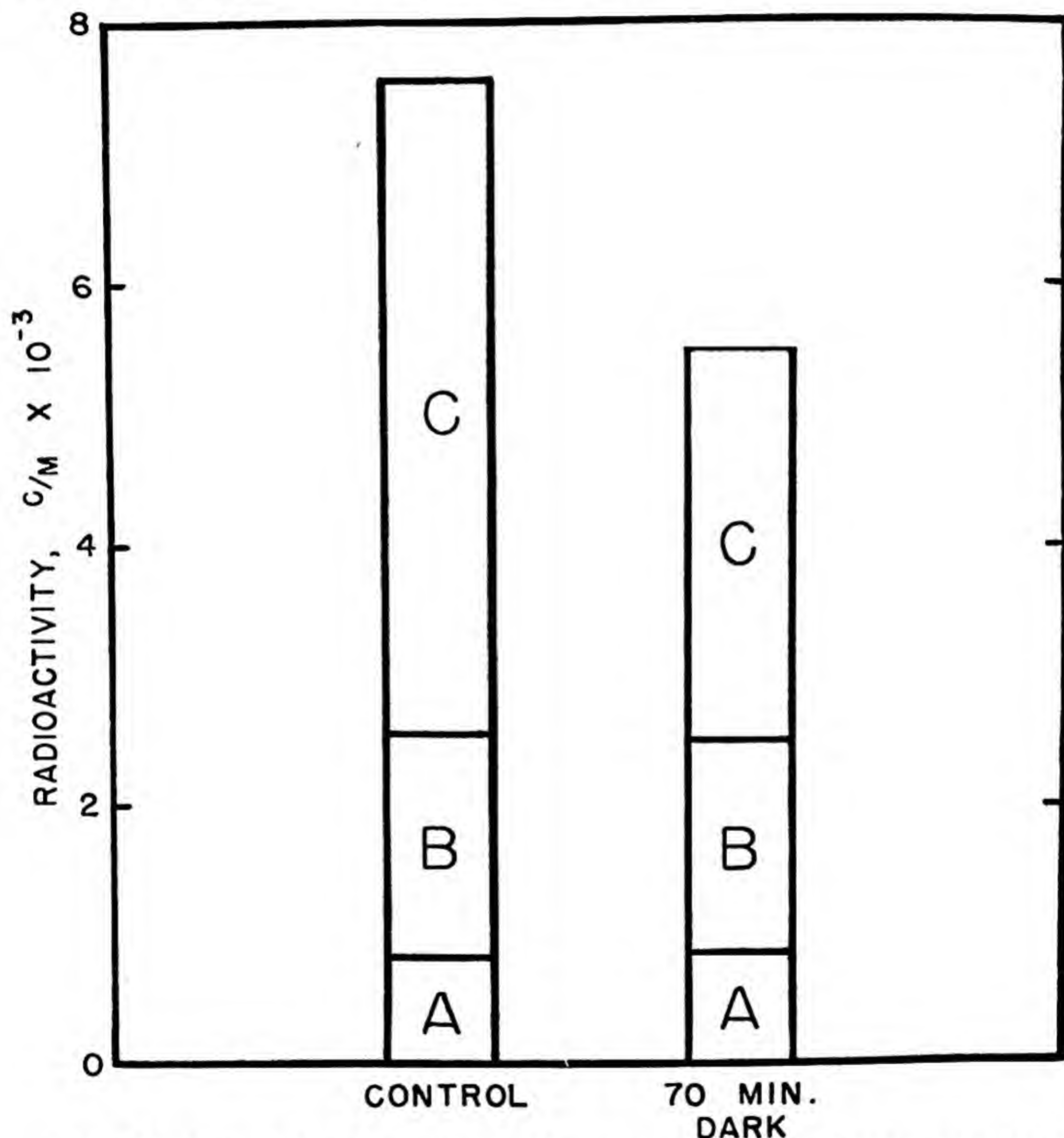


FIG. 20.5—Effect of CO₂ in the dark on tracer previously incorporated by photosynthesis.

reasonable explanation to account for the rapid transformation of "B" in the light as well as for its remarkable stability in the dark is to assume that "B" is not a completely reduced product of photosynthesis but rather a *photosensitive intermediate*, i.e., it must be one of the intermediate compounds which precede the last photochemical reaction in the sequence leading to the first

product. If "B" represents not one but several of these photosensitive intermediates, they must be sufficiently similar as to their chemical nature to be found in the same water-soluble fraction. For simplicity of discussion we shall assume that "B" is one compound.

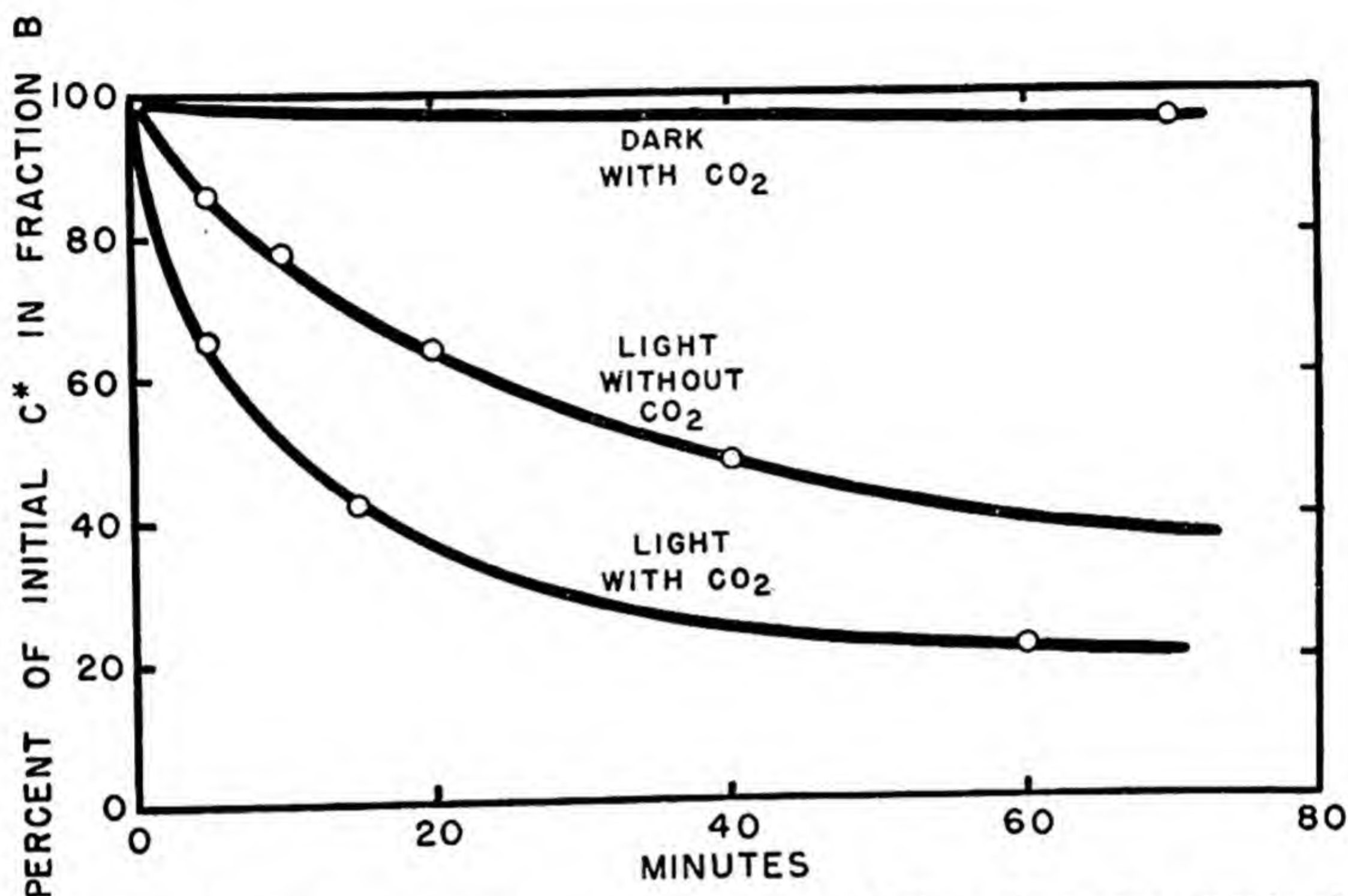


FIG. 20.6—Effect of light and CO₂ on depletion of tracer previously incorporated into fraction B by photosynthesis.

Substance "B" does not exchange tracer carbon with non-radioactive CO₂ in the dark.—If, after tagging "B" by a brief exposure of photosynthesizing algae to tracer CO₂, the cells were kept in the dark in an atmosphere containing ordinary CO₂, no tracer was lost from fraction "B." Figure 20.5 illustrates the stability of tracer in "B" in the course of such an experiment. It is significant that "B," the substance which, according to our fractionation procedures, appears to be the first one to accumulate tracer CO₂ in the photosynthetic reaction sequence, does not have the property usually attributed to the dark fixation product; viz., exchangeability with CO₂ in the dark. To the fact that "B" is subject neither to respiratory nor fermentative transformation, we must add this additional evidence of its stability in the dark.

Transformation of "B" into compounds in other cell fractions depends not only upon light but, in part at least, upon continued photosynthesis; i.e., upon the presence of CO₂.—Figure 20.6 shows that the rate at which tracer is shifted out of fraction B is

negligible in the dark, is appreciable in the light in the absence of additional CO_2 , and is most rapid in the light when photosynthesis is continued with ordinary CO_2 after the tracer has been exhausted. The initial slopes of the two lower curves in Figure 20.6

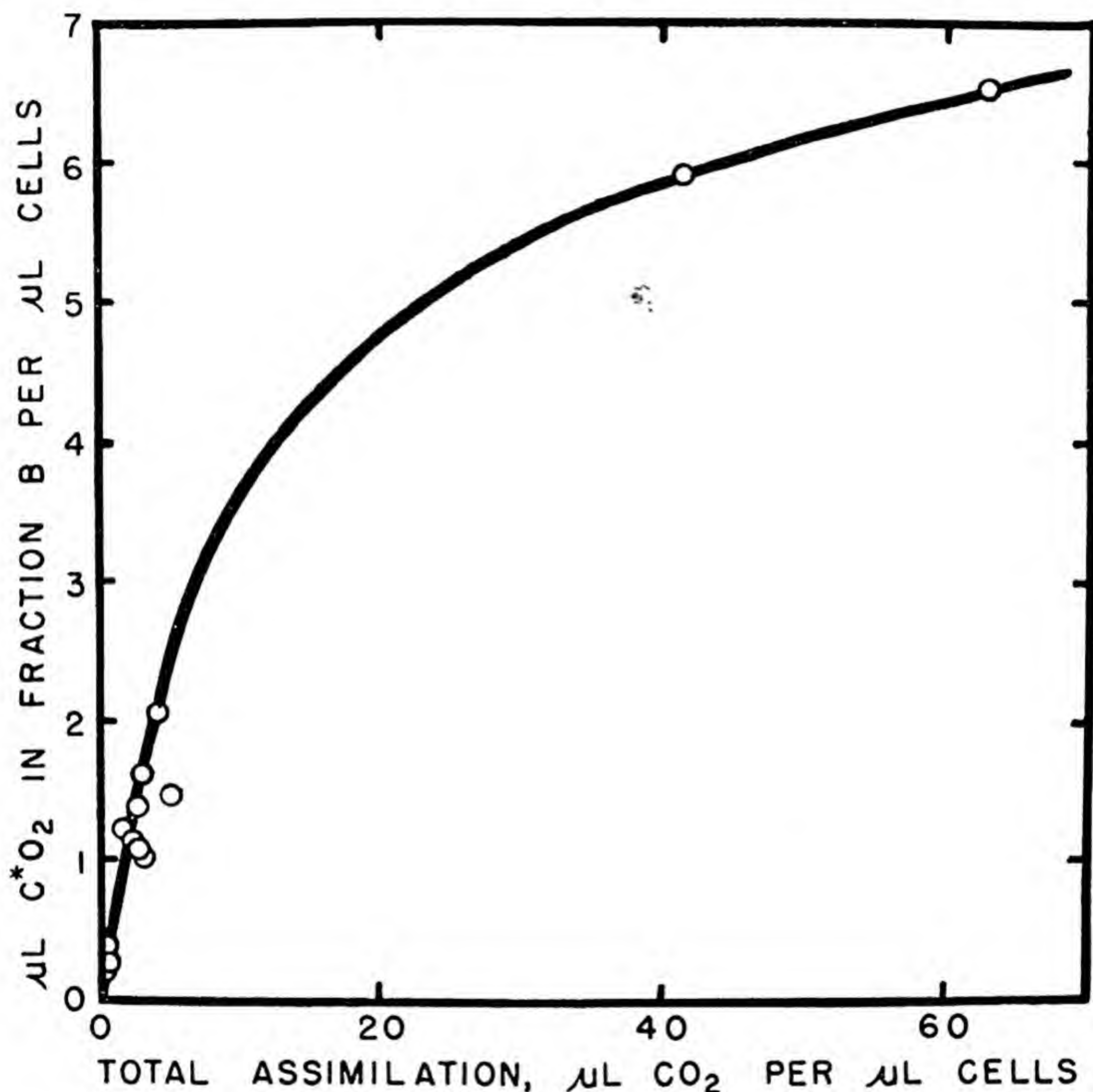


FIG. 20.7—Relation between tracer contained in fraction B and total photosynthetic assimilation.

differ by a factor of four. While, at the present time, we do not wish to offer any particular explanation for the influence of CO_2 on the rate of transformation of "B" in the light, speculative schemes can be suggested which account for the effect without encountering any serious theoretical difficulties.

Saturation of fraction B with tracer carbon occurs only when practically all the carbon of the fraction has been tagged.—

Figure 20.7 shows the results of experiments with different samples of algae which indicate that fraction B becomes saturated with tracer only after a prolonged period of photosynthesis. If we estimate a saturation value from Figure 20.7 and compute the tracer carbon incorporated in fraction B at this point, we can compare this with the total amount of carbon in the fraction. (Fraction B amounts to about 1.7 per cent of the dry weight of the algae. We may assume that its carbon content is approximately 50 per cent.) The calculated amount of tracer and the total amount of carbon are roughly equivalent. Apparently fraction B saturates with tracer only when nearly every carbon atom in the fraction has become tagged. Unfortunately this cannot be taken to mean that "B" is the only substance in this fraction; the fact that "B" can be concentrated manyfold as to counts per milligram demonstrates the nonhomogeneity of the fraction. We find a reasonable explanation of the kinetics of Figure 20.7 if we assume that most of the constituents of this fraction, other than "B," are metabolically very reactive. Since fraction B no doubt contains mostly compounds of low molecular weight (such as members of the tricarboxylic acid cycle), this assumption appears sound. It is to be expected that these compounds should be in equilibrium with substances to be found in other fractions. Tracer first incorporated into "B" would be shifted to other components of this fraction and to other fractions and then, by reactions not directly related to photosynthesis, shifted back again in the form of tagged components of fraction B other than "B" itself. Thus, during prolonged photosynthesis considerable amounts of tracer would be expected to accumulate in fraction B in the form of compounds only very distantly related to photosynthesis.

DISCUSSION AND CONCLUSIONS REGARDING THE LIGHT PRODUCT

The very first reaction in the photosynthetic process should be the formation of the so-called fixation product. If "B" consisted wholly or partly of this carboxylation product, it ought to exchange its tracer readily with non-radioactive CO_2 in the dark. "B" has been shown not to do so. Therefore, we may assume that the dark fixation product required by theory to explain various kinetic phenomena in photosynthesis is unstable (at least with the extraction methods employed). Such an assumption implies that the fractionation procedures which thus far have been employed in tracer studies on photosynthesis are inadequate for investigating the nature of the dark fixation product, since that compound presumably is destroyed during the initial extraction.

The characteristics of the kinetics of formation and transformation of "B" have led us to conclude that this key substance is a

photosensitive intermediate, more likely an early rather than a late member of the reaction sequence. It has, of course, been suggested that the fixation product and the successive intermediates may be represented as different groups on the same "carrier" molecule. This complication would not alter the tracer kinetics as far as short duration tagging experiments are concerned, nor affect the above assumption regarding the instability of the groups acquiring tracer in the dark.

From a consideration of the shape of the curve for fraction *B* in Figure 20.1, it is possible to draw conclusions concerning the number and quantities of intermediates present in this fraction. This curve starts with maximal (negative) slope and, as assimilation of tracer proceeds, its slope continually decreases. This implies that, in the first moment of illumination, some tracer has passed entirely through whatever part of the sequence of photosynthetic intermediates is contained in fraction *B*, and that no appreciable lag precedes the shift of tracer into the other fractions. If several intermediates were contained in fraction *B*, the first would have to accumulate tracer before the second could acquire much; the second would have to accumulate a reservoir of tracer before the next in the series could acquire much; and so on to the last intermediate of the sequence contained in fraction *B*. There would, therefore, be an appreciable lag before tracer could appear in the other fractions. The curve for fraction *B* in Figure 20.1 in such a case would be sigmoid, with its slope at first increasing and later decreasing only as the series of intermediates in this fraction became saturated with tracer. There was no indication of such a sigmoid character in any of our data. From this we conclude that no very large concentration of more than one photosynthetic intermediate is contained in fraction *B*.

INCORPORATION OF TRACER IN THE DARK

Along with our experiments on photosynthesis, dark controls, of course, were necessary. The magnitude of tracer fixation in the dark was so negligible compared with that in photosynthesis that no serious corrections were involved except for photosynthesis at very low light intensity. However, experiments on dark uptake of tracer CO_2 might have a special significance in relation to the "dark pickup reaction."

The kinetic studies of McAlister and Myers (5) provided indirect but convincing evidence of a dark uptake reaction apparently directly related to photosynthesis. They showed that CO_2 continued to be absorbed in the first seconds of a dark period after an illumination of wheat leaves. As is true also of photosynthesis, the "after pickup" was sensitive to CO_2 concentration only at very low partial

pressures of this gas. Aufdemgarten (6), in comparable experiments on a green alga, found that the after pickup was sensitive to cyanide. When Ruben and co-workers (7) reported their observation of a cyanide-sensitive dark fixation of labeled CO_2 by *Chlorella*, it seemed to be a clear demonstration of the reaction which had been postulated for theoretical reasons as the first step in photosynthesis. Their work thus confirmed, by the very sensitive tracer technique, the findings of McAlister and Myers and of Aufdemgarten who observed the uptake of CO_2 only as the consumption of a measurable volume of this gas.

However, in the few years since these papers were published, developments in the field of respiratory physiology have altered materially our interpretation of the kind of result which Ruben *et al.*, and subsequent workers have obtained in tracer experiments on the dark pickup reaction. As early as 1936 Wood and Werkman (8) announced their classic discovery that heterotrophic bacteria may incorporate CO_2 by the reversal of a respiratory decarboxylation. A host of similar observations have since accumulated, and it is now recognized that probably any living cell may fix CO_2 via the reversible decarboxylation reactions involved in respiration and fermentation. While a stream of CO_2 is continuously released from the respiring cell, a small part always re-enters the cell and takes part in a cycle of complicated metabolic reactions. A striking example is the fate of tracer bicarbonate injected into starved rats which are fed lactate at the same time. As much as 16 per cent of the newly formed liver-glycogen is found to be labeled with the tracer (9).

The various reports of dark fixation of CO_2 by bacteria, animal tissues, and non-green as well as green plant cells all serve to emphasize that dark fixation of CO_2 is not peculiar to photosynthesizing cells, but is a very general phenomenon. Therefore, the fact that tracer is incorporated in this or that constituent of a plant does not prove that it has been acquired by photosynthesis, even if previous illumination is found to affect the amount of tracer incorporated. If, for example, in the above mentioned experiments, the rats had been given some indirect stimulation of their metabolism—let us say a strong sun bath—possibly they too would have incorporated even more of the tracer. Despite the apparent relation between irradiation and tracer fixation, such an observation would by no means allow us to conclude that the rats were doing photosynthesis.

It now seems doubtful whether the dark fixation discovered by Ruben *et al.*, in green plants was specifically related to photosynthesis. The same uncertainty applies to most of the elaborate chemical experiments of Benson and Calvin (4). These workers

combined the tracer method with the technique of studying only the after pickup of labeled CO_2 in the dark immediately subsequent to light exposure. Encouraged by the fact that the amount of fixation was increased by previous illumination, they assumed that respiratory intermediates which acquired labeled carbon in their dark fixation experiments must play a role in photosynthesis. Their results seem to have been interpreted without a clear distinction between so-called "photosynthetic" and "respiratory" (or "fermentative") dark fixation of CO_2 . Allen, Gest, and Kamen (10), in an effort to make this important distinction, studied the fixation process in two algal genera in the presence and absence of cyanide. Despite

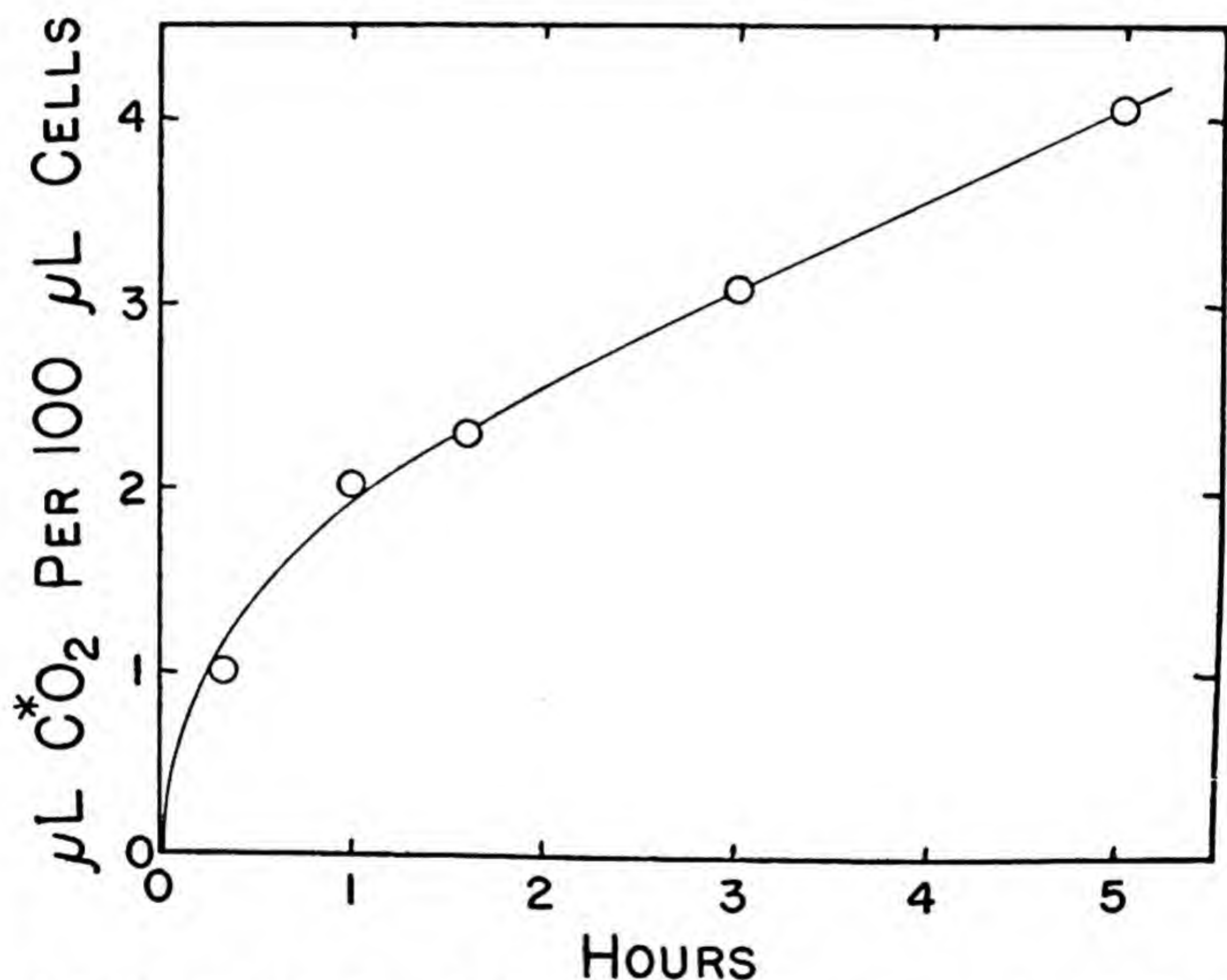


FIG. 20.8—Dark fixation of CO_2 . 105 μl . cells per 5 ml. phosphate buffer in each Warburg vessel. Suspensions exhausted of CO_2 by brief illumination. Tracer added as carbonate solution from side arm. No significant volume uptake of CO_2 observed in any vessel during dark period.

a nearly complete inhibition of respiration in the presence of cyanide, an impressive amount of carbon tracer continued to be incorporated in the dark. As we shall see presently, even this constitutes no proof that the fixation has any relation to photosynthesis. The results of our own observations of the dark uptake of CO_2 in

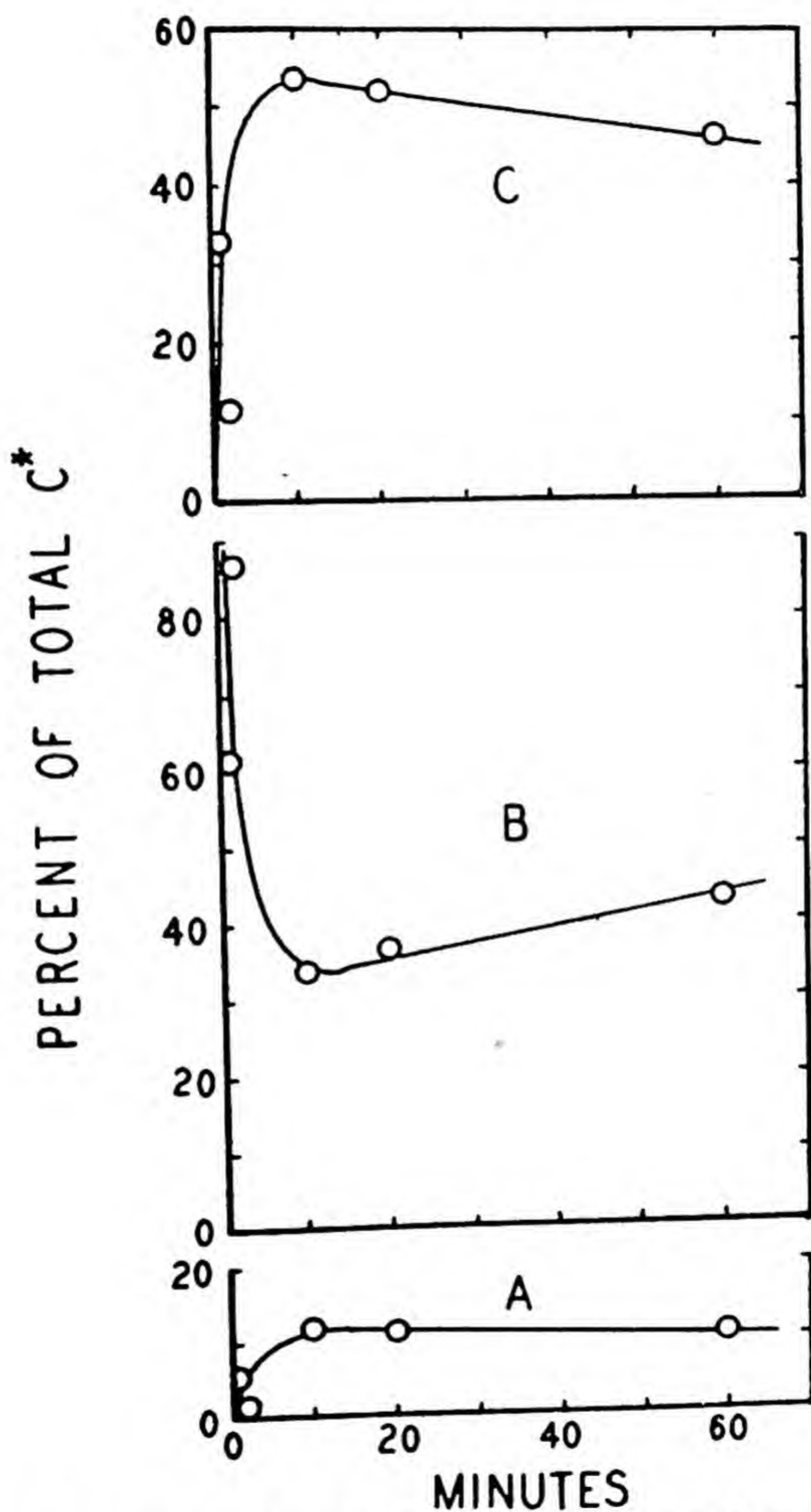


FIG. 20.9—Time course of distribution of tracer CO₂ fixed aerobically in the dark. Each point represents the tracer fixed in a particular chemical fraction of the algae as a percentage of the total tracer incorporated by all fractions.

Scenedesmus force us to state that it cannot be conclusively linked to photosynthesis. On the contrary, we present them here in support of our belief that a tracer experiment demonstrating in a convincing fashion a dark uptake reaction specifically related to photosynthesis has not yet been designed.

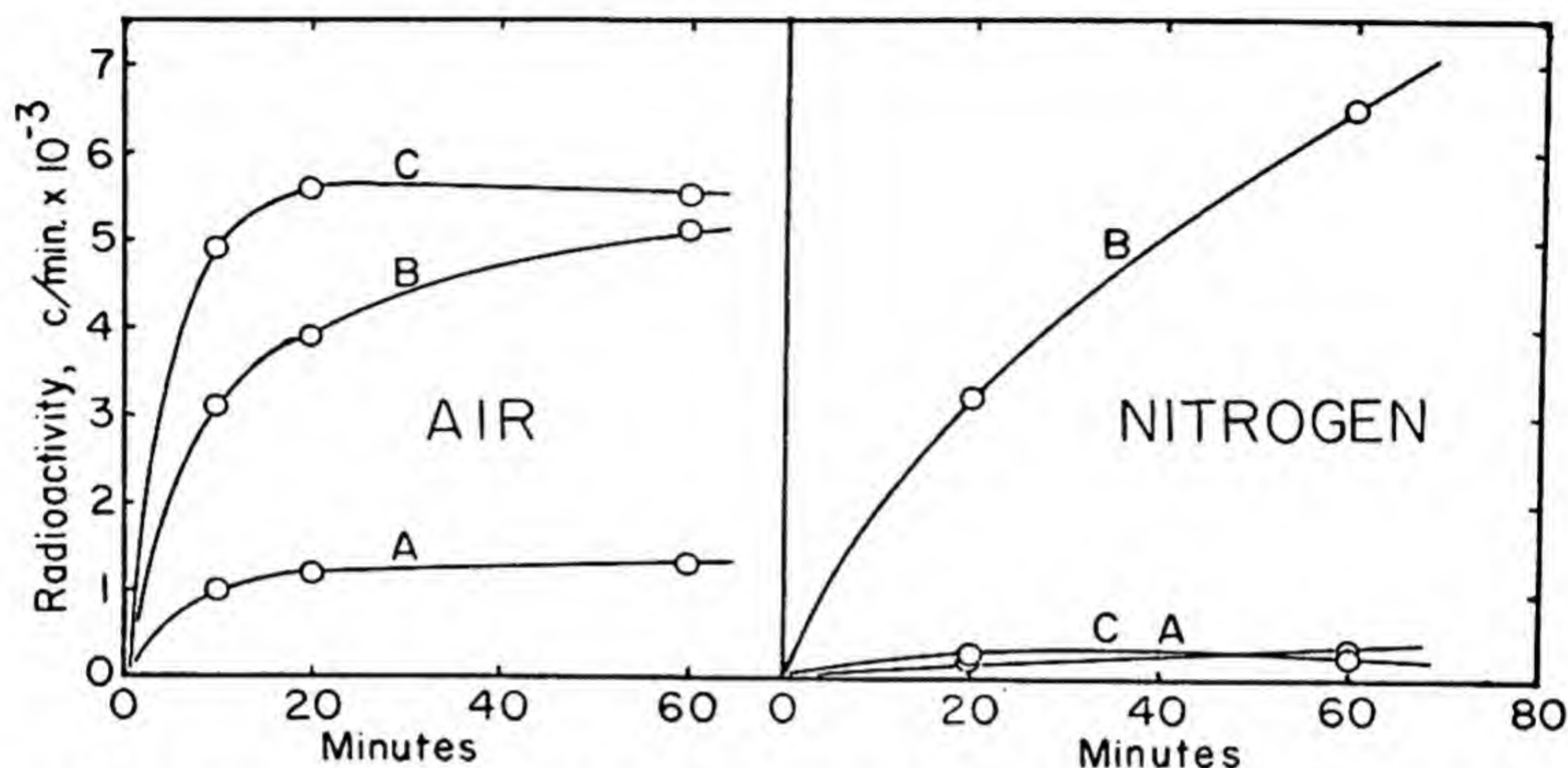


FIG. 20.10—Time course of dark fixation of tracer CO_2 in air and in nitrogen. Letters refer to fractions into which the algal constituents were partitioned. For characteristics of fractions see Figure 20.2.

Figure 20.8 shows that *Scenedesmus* incorporates tracer CO_2 at a measurable rate for many hours in the dark. The uptake is most rapid initially, though at all times far below the usual rate of assimilation in the light. The rate of uptake in air is about twice the anaerobic rate. When the cells were fractionated (as described for our experiments on photosynthesis), it was found that the different fractions had accumulated tracer at different initial rates. Figure 20.9 suggests that fraction B was the first to acquire tracer; a situation comparable to that encountered in experiments with illuminated algae. The conversion of tracer in this fraction into compounds in the other fractions was found to proceed only aerobically, not anaerobically (cf. Fig. 20.10). Also, tracer incorporated into fraction B in the dark was found to be readily exchangeable with ordinary CO_2 . Figure 20.11 demonstrates these latter characteristics of the dark fixation products and suggests that the aerobic conversion of tracer from fraction B to other fractions is a much more rapid reaction than is the exchange responsible for incorporating tracer initially.

Our experiments in the light and in the dark show in common that tracer apparently is first fixed in fraction B, but here the simi-

larity ends. The dark product readily exchanges tracer with unlabeled CO_2 ; the light product (called substance "B" above) does not. The dark product undergoes very rapid aerobic transformation into compounds in other fractions; the light product does not. The dark product is respirable to CO_2 in an atmosphere free of this gas; the

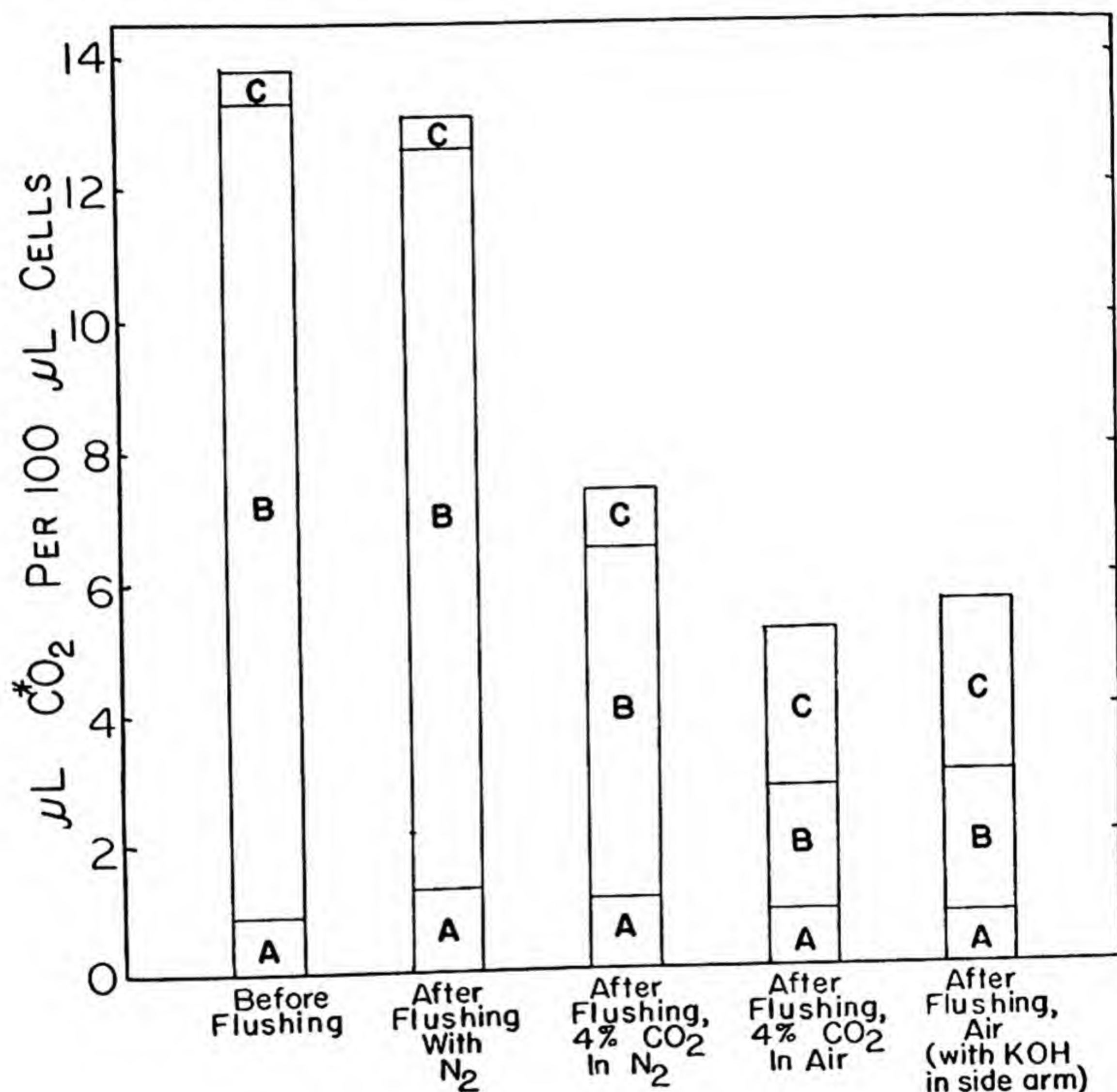


FIG. 20.11—Transformations of products of dark fixation of CO_2 .

light product is not. The dark product appears to be formed chiefly by exchange reactions not involving a net uptake of gas, and has counterparts in many non-photosynthetic cells of plants and animals. The light product is most specifically involved in photosynthesis and, as we have pointed out above, its kinetic behavior is that of an intermediate in the sequence of photosynthetic reactions.

SUMMARY

Green plants distribute tracer carbon assimilated by photosynthesis into numerous cell constituents in a matter of minutes. If photosynthesizing cells of *Scenedesmus* are exposed to CO_2 (labeled with C^{14}) for periods not exceeding forty seconds, most of the assimilated tracer is found in a small water-soluble fraction. The substance containing tracer in this fraction is not affected by respiration or fermentation, nor is its tracer carbon exchangeable in the dark with inactive CO_2 . It is transformed by exposure to light, whether in the presence or absence of additional CO_2 . The kinetics of its formation and transformation and its properties indicate that it is a photosensitive intermediate on the path between the primary dark fixation product and the final finished product of photosynthesis. Chemical investigation of this substance is discussed in the following paper.

The product of dark fixation has very little in common with the product of light fixation, and it is suggested that it may have no connection with photosynthesis.

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21.

Investigation of the Chemical Properties of Intermediates in Photosynthesis

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AS THE preceding paper has shown, an investigation of the kinetics of the fixation of tracer carbon during photosynthesis in the alga, *Scenedesmus obliquus*, reveals the following facts: (a) The incorporated CO₂ first appears in a chemically and thermally stable form in that fraction which can be extracted from the algae by boiling water. Over 85 per cent of the total activity fixed by the algae is found in this fraction, when the period of exposure to radioactive carbonate at near-saturating light intensities is of less than sixty seconds duration. (b) The major portion of the tracer carbon fixed during short periods of photosynthesis should be present in only one or two compounds. (c) Tracer carbon fixed during short periods of photosynthesis is exceedingly resistant to movement into the rest of the cell by general metabolic reactions. It is, however, subject to further photochemical transformation, and must therefore be considered an intermediate in the process of photosynthesis.

Consideration of the above facts, coupled with the seemingly reasonable expectation that photosynthesis should involve somewhat the same intermediates as does the chemosynthesis of carbohydrates, makes the isolation of the photosynthetic intermediate(s) by the application of tracer methods appear to be a simple task. It has, however, proven to be unexpectedly complex. Not only is the fraction not quite so homogeneous as to tracer as predicted, but none of the known intermediates of sugar metabolism has been found to contain activity in significant amounts. This has made it neces-

¹ Frank B. Jewett Fellow, 1946-1947.

sary to proceed by a stepwise investigation of one class after another of substances in order to eliminate each as a possible intermediate.

MATERIALS AND METHODS

Growth and tagging.—The plant material used in this investigation is the alga, *Scenedesmus obliquus*, strain *D*₃, grown on inorganic medium at 30°C. and at a pH of 8. Using a 100 liter tank, approximately a pound of algae is produced per week. Small aliquots of these algae, suspended in phosphate buffer, are allowed to use up essentially all external and internal CO₂ by photosynthesis. Sodium carbonate solution containing C¹⁴ is then added and exposure to light is continued for periods of less than a minute. At the termination of this period the algae are killed by pouring the suspension into boiling water. The aqueous extract from these algae is added to a similar extract from the bulk of the algae. The use of this natural carrier material reduces the chance for error in conclusions due to coprecipitation, adsorption on precipitates, etc., inevitably introduced by the addition to such a complex mixture of foreign substances of similar properties but not identical with the tagged substance.

Fixation of CO₂ in the dark proceeds at such a low rate compared to fixation by photosynthesis that its contribution is entirely negligible when exposure of the algae to tracer at near-saturating light intensities is limited to short periods. However, it can become a considerable factor if low light intensities or their equivalent, very dense suspensions of algae, are used in the tagging process. All of the work reported in this paper has been carried out with algae exposed to radioactive CO₂ at near-saturating light intensities for periods of less than one minute; the usual period being thirty to forty seconds.

Measurement of radioactivity.—The radioactivity is determined with a mica-window Geiger counter and a conventional scaling circuit. All determinations are made with the same geometry and the same air and window absorption. Therefore, only relative activities are measured, and the only corrections applied are for self-absorption in the material being counted. Solids are counted in aluminum dishes 1 cm.² in area and, if sufficient material is available, at over the maximum range of 20 mg./cm.² Materials in solution are counted by evaporating an aliquot of the solution in a small aluminum dish using a disk of lens paper to facilitate spreading of the sample. Extensive tests have shown that both methods give results consistent to within ± 5 per cent, exclusive of the statistical error.

GENERAL CHARACTERISTICS OF THE AQUEOUS EXTRACT

The aqueous extract contains an average of 1.7 per cent of the dry weight of the algae; the pH is within a few tenths of neutrality. It shows no fluorescence in the visible when exposed to ultra violet light.

Color.——The solution is yellow in color. The color is discharged by the addition of reducing agents (sodium hydrosulfite or zinc and acid) and reappears when such a decolorized solution is shaken with air. The solution in its oxidized form gives a very faint test for ferric ion with excess thiocyanate and none at all with ferrocyanide. The reduced solution, however, gives a strong test for ferrous ion with α,α' -dipyridyl. The color and the difficulty of obtaining a test for ferric ion suggest the presence of a very stable complex involving ferric ion and a polyhydroxy acid.

Volatility.——No radioactivity is lost by evaporation of the water extract under acid, neutral or basic conditions, or by steam or vacuum distillation. Although methyl and butyl esters can be prepared, as is shown by the solubility of the activity in chloroform-alcohol mixtures after treatment with the anhydrous alcohols and hydrogen chloride, they cannot be distilled undecomposed under high vacuum. These observations remove as possibilities all of the volatile aldehydes, alcohols, acids, and ketones.

Decarboxylation.——The solid resulting from vacuum drying of the aqueous extract evolves activity in the form of CO_2 when heated to 220°C . in quinoline. When no catalyst is used, about 10 per cent of the available activity is evolved. Addition of a copper chromite catalyst increases this to almost 30 per cent. No appreciable change in these percentages occurs if the material has been subjected to acid hydrolysis previous to the vacuum drying. Visual evidence suggests that the gas evolution does not start until a temperature above 195°C . is reached. These results, in conjunction with the evidence for esterification (cf. Volatility), prove that some of the activity is present in the form of carboxyl groups. However, it is surprising that after such short periods of photosynthesis only 30 per cent of the entering CO_2 should still be in the form of carboxyl.

Stability toward acids.——All attempts at hydrolysis with mineral acids have led to the formation of considerable insoluble material which fortunately carries down very little activity. Although refluxing with 6 N H_2SO_4 or HCl or 2 N HNO_3 causes a darkening of the solution to deep brown betokening decomposition, less than 5 per cent of the activity is lost in volatile products. No additional

loss occurs when such acid hydrolysates are evaporated to small volume under vacuum.

Solubility.——The active material is exceedingly soluble in water, relatively insoluble in anhydrous alcohols except in the presence of hydrogen chloride and almost completely insoluble in all other common organic solvents. Long continued extraction of the acidified aqueous extract with ether or ethyl acetate transfers approximately 5 per cent of the activity into the organic solvent. It is entirely possible that this represents entrainment of the water solution by the organic solvent. Between 15 and 20 per cent of the activity is transferred into butanol when water solutions (0.5 N as to H_2SO_4) are shaken with this solvent. Continuous extraction by boiling butanol of a neutral or basic solution of the water extract removes very little activity; continuous extraction of the acidified extract removes about the same percentage as does shaking; continuous extraction of acid hydrolyzed material removes about 80 per cent of the activity along with most of the color. The latter extraction has not been extensively employed because of the decomposition caused by acid hydrolysis and the occurrence of varying amounts of esterification during the extraction.

Adsorption.——In order that the photochemical reduction process may take place efficiently it is necessary that the substance being reduced be adsorbed on the chlorophyll-protein surface (cf. paper by Franck, this volume). Thus any intermediate in photosynthesis, subject to further photochemical transformation, must be strongly adsorbed on active surfaces. Such is indeed the case with the material into which tracer is incorporated during illumination periods of less than a minute. It is completely removed from solution in 50 per cent ethanol by carbon (Darco G60) or Fuller's earth (Florex xxx) and no effective solvent for elution from these adsorbents has been found.

The formation of precipitates in a solution containing the active material results in the adsorption of varying amounts of activity on their surfaces. Barium sulfate and phosphate seem to be especially troublesome in this respect. The activity can seldom be washed from the precipitate, re-solution and reprecipitation being the only practical means for its removal. In many cases a choice has to be made between having sufficient material to give reliable results in counting and the removal of the last few per cent of activity from a precipitate. Because of this, when small amounts of precipitate are obtained from solutions of high activity, it has been assumed that association of 5 per cent or less of the available activity with the precipitate is due to adsorption and has little significance.

The usefulness of the Amberlite ion-exchange resins is much reduced by this tendency toward general adsorption. Activity is retained by both the anion and cation resins but the amounts retained are variable. Approximately 30 per cent of the activity is not recoverable from the resins by elution. Thus it was felt that, while ion exchange was surely occurring, it was complicated by nonselective adsorption.

TESTS FOR SPECIFIC CLASSES OF COMPOUNDS CARBOHYDRATES

Although its stability against metabolic transformation and its sensitivity to further photochemical transformation make it appear very unlikely that the intermediate into which tracer is incorporated during short periods of illumination is a sugar, this possibility had to be investigated. Of the sugars, the simplest type, a triose, might be thought most likely to occur. With this in mind, the following experiment was performed. Ten ml. of 34 N H_2SO_4 were added to 500 ml. of the aqueous extract from the algae, the solution was distilled to a volume of 50 ml. (6.8 N as to H_2SO_4) and the distillation continued, with the addition of water to maintain the volume at 50 ml., until 300 ml. additional distillate had been collected. The dinitrophenyl hydrazones were prepared from the combined distillates (1). The hydrazone precipitate contained only 3.6 per cent of the available activity (1,515 cpm. out of 42,100 cpm.). The sulfuric acid solution was shown to contain over 90 per cent of the original activity. Under these conditions glyceraldehyde, dihydroxyacetone, and their phosphate esters are known to be converted to the volatile methyl glyoxal, its dinitrophenyl hydrazone being obtained in over 90 per cent of the theoretical yield (2). Refluxing the aqueous extract from the algae (made 2 N as to HCl) with dinitrophenyl hydrazine yielded an appreciable precipitate, but only 2-3 per cent of the available activity was present in this precipitate. These experiments, coupled with repeated observations that the loss of activity as volatile products during acid hydrolysis and subsequent vacuum evaporation is always less than 5 per cent, make impossible the presence of any triose containing more than trace amounts of radioactive carbon.

Pentoses and hexoses were eliminated early in the investigation by three separate methods. When the phenyl osazones were precipitated under conditions giving 68 per cent of the theoretical yield of glucosazone, the precipitate was completely devoid of activity after two recrystallizations. The same result was obtained when glucose was added as carrier. Oxidation by periodic acid formed no volatile products containing activity, though in one experiment both the formaldehyde and formic acid from added glucose were recovered

in 90 per cent of the theoretical yield. When acetylation was performed with acetyl bromide, followed by extraction with benzene, under conditions which gave 80–85 per cent of the theoretical yield of the benzene-soluble polyacetates of the sugars, less than 5 per cent of the activity was retained in the benzene. This residual activity may well have been the result of insufficient washing of the benzene solution with water.

The intermediate in which photosynthetically incorporated CO_2 first appears in a chemically and thermally stable form is not a carbohydrate.

AMINO ACIDS

The aqueous extract gives a strong ninhydrin test for alpha amino acids and a weak biuret reaction. Paper chromatography indicates the presence of alanine, glutamic acid, and aspartic acid as the major amino acid constituents. The amount of protein is small, for only a tiny precipitate is obtained when trichloroacetic acid is added to the extract. When the aqueous extract, either untreated or acid hydrolyzed, is heated with ninhydrin in phosphate buffer the evolved CO_2 contains no activity. Added glycine or alanine gave a quantitative yield of CO_2 under these conditions. The benzoylamino acid precipitate obtained by benzoylation of the aqueous extract, either untreated or acid hydrolyzed, with benzoyl chloride in the presence of NaOH, sodium bicarbonate, or pyridine (on dried solids from vacuum evaporation of extract) never contained more than 5 per cent of the available activity, although in one experiment 70 per cent of the theoretical yield of benzoyl alanine was obtained from added alanine. However, some benzoylation of active material did occur for, after benzoylation, about 15 per cent of the activity could be extracted by chloroform or carbon tetrachloride from strongly acid solution, whereas these solvents would extract no activity previous to this treatment. Table 21.1 presents the results of a typical benzoylation experiment.

KETO ACIDS; DICARBOXYLIC ACIDS

The metabolic inertness of the substance containing the tracer carbon makes it unlikely that it is present in the form of any of the usual components of dark fixation reactions. Its presence in such a form is rendered even less likely by its sensitivity to further photochemical transformation. This prediction from kinetics is fully substantiated by investigation of the chemical properties of the substance.

The keto acid components of dark fixation are removed as possibilities by the failure to obtain any significant amount of activity in the precipitate formed with dinitrophenyl hydrazine and

by repeated observation that less than 5 per cent of the activity is lost during prolonged hydrolysis with 6 N mineral acids (cf. Carbohydrates). Even heating to 120°C. with syrupy phosphoric acid caused no loss of activity as CO₂.

None of the very sensitive color reactions for citric, tartaric, glycolic, or malic acids could be obtained on material which had been concentrated more than tenfold as to specific activity (cf. Purification). In the same material an active preparation of succinic

TABLE 21.1
BENZOYLATION

Fraction	Percentage Available Activity	Weight
Soluble in CCl ₄	13.4	173 mg. (mostly benzoic acid)
Soluble in acid	90.6	454 mg. (mostly sodium chloride)
Insoluble in acid	2.3	36 mg.
	106.3	

Activity available: 1960 cpm.

Activity recovered: 2083 cpm.

Reagent: Benzoyl chloride + NaOH.

dehydrogenase (obtained through the courtesy of Dr. B. Vennesland, Dept. of Biochemistry, University of Chicago) failed to show the presence of any succinic acid. Although the purification procedure suggested the presence of the sodium salt of an organic acid, all attempts to form *p*-nitro benzyl or *p*-bromo phenacyl esters failed. In no case was more than 5 per cent of the available activity extracted by ether from the reaction mixture. The fact that continuous extraction with ether or ethyl acetate does not extract any appreciable activity is further powerful evidence against the presence of tracer in any of the acids involved in dark metabolism; oxalic, malic, tartaric, glycolic, lactic, pyruvic, succinic, citric, etc., (cf. Solubility).

Partition chromatography on silica gel with 0.5 N H₂SO₄ as the stationary aqueous phase and 50/50 butanol-chloroform as the mobile organic phase (3) revealed that somewhat over 20 per cent of the activity in the extract from the algae was less strongly retained by the aqueous phase than was tartaric acid. However, the major portion of the activity was almost completely retained by this phase. Acid hydrolysis prior to the chromatography had a negligible effect on this distribution. Table 21.2 presents the results

of the application of partition chromatography to the aqueous extract, both untreated and acid hydrolyzed. The distribution of tartaric acid under identical conditions is included for comparison.

The intermediate in which photosynthetically incorporated CO_2 first appears in a chemically and thermally stable form is not one of the keto acids or dicarboxylic acids associated with dark fixation of CO_2 .

TABLE 21.2
SILICA GEL CHROMATOGRAPHY

Fraction	Percentage Available Activity Recovered		Percentage Tartaric Acid Recovered
	Non-hydrolyzed	Hydrolyzed	
1.....	22.7	23.6	10.1
2.....	3.4	2.7	70.0
3.....	1.3	2.7	16.6
4.....	1.0	2.4
Retained on SiO_2 .	70.0	65.1
Total.....	98.4	96.5	96.7

Activity available: 1357 cpm.

Activity recovered: Non-hydrolyzed-1334 cpm.

Hydrolyzed-1308 cpm.

Organic phase: 50/50/butanol/chloroform collected in 50 ml. fractions.

Aqueous phase: 0.5 N H_2SO_4 .

POLYHYDROXY ACIDS

Failure to identify the major portion of the activity with any of the above classes of compounds, coupled with its extreme water solubility and the indication of the presence of a polyhydroxy acid complexed with ferric ion (cf. Color), led to the suggestion that the active substance might be a polyhydroxy acid or a phosphate ester of such an acid.

The simplest representative of this class of compounds is glyceric acid. An investigation of this substance and its phosphate esters was in progress when the paper of Calvin and Benson (4) appeared. Because our results are in complete disagreement with theirs, this matter will be discussed in some detail. Two methods of attack have been used; (a) isolation of the phosphate esters as barium salts, and (b) acid hydrolysis followed by oxidation with periodic acid, with isolation of the formaldehyde as its dimethone compound and of the glyoxylic acid as its bicarbonate-soluble dinitrophenyl hydrazone.

The aqueous extract from the algae was evaporated to small

volume under vacuum, an equal volume of ethanol was added, and the solution was made acid with acetic acid. The barium salts were precipitated by the addition of excess barium acetate to this solution (5). The original precipitate was dissolved in dilute HCl and reprecipitated by the addition of sodium acetate, acetic acid, and varying amounts of alcohol. This procedure has been carried out both with and without added 3-phosphoglyceric acid (from yeast). The original precipitate contained about 20 per cent of the total activity present in the solution. Purification by reprecipitation from lower alcohol concentrations indicates that the activity, if it is in a phosphoglyceric acid at all, is in the 2-isomer whose barium salt is reported to be more soluble in aqueous alcohol than is that of the 3-isomer.

The results of a typical experiment with added 3-phosphoglyceric acid are shown in Table 21.3. Information as to the percentage recovery of barium 3-phosphoglycerate dihydrate to be expected under such conditions is included at the bottom of the table. It will be especially noted that so long as the amounts of precipitate were large and the conditions of precipitation were not altered the specific activity remained constant. However, the relative specific activities of *C* and *D* make it obvious that the precipitate was far from homogeneous. It has been found that reprecipitation of substances under conditions identical with the initial precipitation often gives spurious evidence of homogeneity, i.e., constant specific activity. This evidence is only shown to be false when the conditions of precipitation are changed significantly.

The very great tendency of the active material toward adsorption and coprecipitation can scarcely be overemphasized. The precipitate in *C* was crystalline, showed all the properties of the added 3-phosphoglyceric acid, and accounted for 72 per cent of the weight of this material. However, only 7 per cent of the available activity and only one-third of the activity present in the initial precipitate was associated with it. A comparison of *C* and *D* suggests that even this small residual activity might be removed by repetition of the process.

The second method of attack, involving oxidation by periodic acid, was carried out as follows: The aqueous extract from the algae was subjected to acid hydrolysis under varied conditions of acid strength and temperature. The hydrolysate was adjusted to pH 7 and periodic acid was added until a test showed a slight excess. After standing thirty minutes at room temperature, the excess of periodic acid was removed with bisulfite, and the solution was subjected to steam distillation. Formaldehyde was isolated from the distillate as the dimethone compound and glyoxylic acid was isolated

from the solution as the bicarbonate-soluble dinitrophenyl hydrazone. No activity was ever observed in the volatile fraction, nor, as shown by the combined recoveries from the other fractions, was any to be expected. Experiments were performed both with and without added glyceric or 3-phosphoglyceric acids. The amount of hydrazone obtained when no carrier was added was negligible.

TABLE 21.3
PRECIPITATION OF BARIUM SALTS

Precipitation	Solvent	Percentage Available Activity Precipitated	ct/mg.	Weight
(A) from original solution	50 per cent ethanol (20 ml.)	20.5	17.1	150 mg.
(B) from solution of preceding precipitate	50 per cent ethanol (20 ml.)	16.7	17.9	120 mg.
(C) from solution of preceding precipitate	20 per cent ethanol (6 ml.)	7.1	12.6	72 mg.
(D) by addition of more alcohol to filtrate and washings from preceding precipitate	50 per cent ethanol (11 ml.)	2.4	33.2	9 mg.

Weight of Ba phosphoglycerate. $2\text{H}_2\text{O}$ dissolved in HCl: 100 mg.
Solvent: 50 per cent ethanol (20 ml.)
Weight of precipitate: 95.4 mg.
Percentage recovery: 95.4

Activity available: 12,950 cpm.
Carrier added: 100 mg. of barium phosphoglycerate dihydrate.

The results of typical experiments with added glyceric and 3-phosphoglyceric acid are shown in Table 21.4. These results suggest that if any activity at all is present in the form of glyceric acid or one of its phosphate esters, it is confined to the carboxyl group and the alpha carbon atom. However, careful purification of the combined hydrazones from several experiments by solution in bicarbonate and fractional precipitation with acid reduced the specific activity from twenty counts per mg. to six counts per mg. Thus, even the small percentage of activity observed in the crude hydrazone precipitate probably represents only adsorption.

It could, of course, be argued that the 2-phosphoglyceric acid would act in an entirely different manner when subjected to acid hydrolysis than does the 3-isomer. It is possible to assume that it would more easily lose a molecule of phosphoric acid. However, the result of such a loss would be the enol of pyruvic acid which would appear either as the dinitrophenyl hydrazone of pyruvic acid

TABLE 21.4
PERIODIC ACID OXIDATION

Carrier Added	Hydrolysis Conditions	Percentage Yield of Hydrazone *	Percentage Available Activity Precipitated as Hydrazone	Percentage Available Activity not Precipitated as Hydrazone
(A) Glyceric acid.....	1 N HCl 15 hrs./100° C.	96	4.8	95.4
(B) 3-Phosphoglyceric acid.....	6 N HCl 6 hrs./125° C.	49	4.7	93.6

Activity available: (A) 16,187 cpm.

(B) 14,093 cpm.

Activity recovered: (A) 16,196 cpm. (100.2 per cent)

(B) 13,829 cpm. (98.3 per cent)

* Yield based on amount of carrier added.

or, more likely, as the breakdown products of pyruvic acid which would be volatile with steam.

Any higher molecular weight polyhydroxy acid (or phosphate ester) in the same homologous series as glyceric acid would give glyoxylic acid, formaldehyde, and varying amounts of formic acid when oxidized by periodic acid. The latter two products would be volatilized with steam, and the glyoxylic acid would be found in the hydrazone precipitate.

When the aqueous extract from the algae was warmed with acetic acid and phenylhydrazine in a small volume of solution, the precipitate contained less than 5 per cent of the available activity. Gluconic acid added in one experiment gave a 93 per cent yield of its phenylhydrazide without increasing the percentage of activity found in the precipitate.

A seven-hour reflux with concentrated hydroiodic acid (47 per cent) and red phosphorus of the solids obtained by vacuum drying of the aqueous extract from the algae caused the evolution of some CO₂, but it was entirely inactive. Steam distillation of the resulting solution volatilized less than 3 per cent of the available activity. If the activity had been present in a low molecular

weight polyhydroxy acid, it should have been recovered as the corresponding steam-volatile aliphatic (or iodo-aliphatic) acid.

The intermediate in which photosynthetically incorporated CO_2 first appears in a chemically and thermally stable form is not a polyhydroxy acid nor a phosphate ester of such an acid.

PURIFICATION

Attempts at purification of the active material have been only moderately successful. The procedure which has led to the greatest increase in specific activity is outlined below. The aqueous extract was evaporated to dryness under vacuum at low temperature, and the resulting solid was dried over phosphorus pentoxide. This dry powder was shaken for ten hours at room temperature with dry butanol containing hydrogen chloride (0.5 N). The butanol extracted 76 per cent of the available activity; the specific activity increased from 9.6 counts to 17.9 counts per mg. The solvent was evaporated under vacuum, the residue was taken up in water, adjusted to pH 8 with NaOH, and warmed for a short time in a water bath under nitrogen. Both odor and indicator paper gave evidence of the evolution of ammonia during this treatment. A copious precipitate which appeared at this point was centrifuged off and washed. Its specific activity was very low. The addition of alcohol to a concentration of 60 per cent and cooling caused the formation of a crystalline precipitate of somewhat higher specific activity; analysis suggested sodium phosphate containing a small amount of organic material.

After removal of the latter precipitate, the alcohol concentration was increased to 80 per cent and the solution was kept at 0°C . overnight. A brown, gummy material was deposited on the bottom of the flask. This material dried to an exceedingly hygroscopic brown solid. The specific activity was 138 counts per mg., an increase of sixteenfold when corrected for sodium content. The total activity present in this material represented 22.7 per cent of that present in the original aqueous extract.

Although the increase in specific activity was encouraging, it was felt that the low yield in terms of available activity and the evidence of decomposition shown by the very dark brown coloration of the butanol extract were difficulties which should be avoided if possible. Therefore, attempts were made to improve upon the yield and to reduce the evidence of decomposition. All such attempts at improvement have been complicated by two difficulties; the very great adsorptive tendency of the active material, and the extreme sensitivity toward mineral acids of the aqueous extract. It has become apparent that improvement of this method will be very difficult.

Therefore, it is being repeated on a considerably larger scale in order that sufficient material of high specific activity may be obtained to carry out extensive tests.

All information so far obtained indicates that at least two substances containing tracer carbon are present in appreciable quantity in the aqueous extract. It seems entirely possible that the 15–20 per cent of activity which is precipitated by barium ion from 50 per cent alcohol, is extracted by chloroform after benzylation, is extracted by butanol from 0.5 N H_2SO_4 by shaking, and is in the first fraction of solvent to come through the silica gel column, represents one substance or class of substances. Its properties suggest that it is not too complicated, and we have perhaps been incorrect in directing most of our attention to the major fraction which has so far shown such a complete lack of any specifically indicative properties. However, this latter fraction, because it constitutes almost four-fifths of the total photosynthetically incorporated tracer, is certainly of the greater importance.

The aqueous extract from the algae has an absorption peak at 2,600 Å. Although there is not an exact correspondence, this peak is sharpened and the specific absorption is increased when the specific activity is increased by purification procedures. This suggests that a pyrimidine or a purine may be associated with the photosynthetic intermediate(s).

SUMMARY

A study has been made of the chemical properties of the fraction in which photosynthetically incorporated CO_2 first appears in a chemically and thermally stable form in the alga, *Scenedesmus obliquus*. Evidence is presented to show that this fraction, the aqueous extract from the algae, contains two substances into which significant amounts of tracer have been incorporated during periods of exposure to light and tracer carbon of less than sixty seconds duration. It has been shown that the substance containing over 75 per cent of the incorporated tracer is not a carbohydrate (or phosphate ester of such a compound), an amino acid, a keto acid, any acid of the metabolic cycle, an aldehyde, ketone, phenol or alcohol, or a polyhydroxy acid (or phosphate ester of such a compound). The portion containing the remaining 25 per cent of the incorporated tracer has not been so thoroughly investigated. All indications are, however, that it too does not belong to any of the above classes of compounds.

This paper should be considered as a progress report. It is presented in such detail at this time only because of the complete and astonishing disagreement between the results of the work done in

this laboratory and that done at the University of California (4, 6).

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22.

The Comparative Biochemistry of Photosynthesis—

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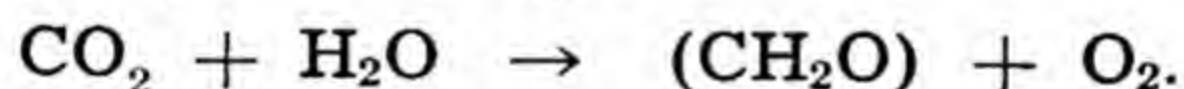
“Il faut reconnaître que ce n'est pas seulement la partie colorée de la plaste qui prend part au processus de la photosynthèse, mais aussi sa partie incolore.”—V. N. LUBIMENKO

BEFORE 1913, the date of publication of Willstätter and Stoll's masterly treatise on chlorophyll (1), little was known concerning the chemical nature of the green pigments of the plant kingdom. The previously collected information was not only cursory and incomplete, but also highly contradictory. The sustained efforts of Willstätter and Stoll brought this period to a close. From that time on the structure of this most important among the naturally occurring coloring matters may be considered as firmly established in its essential features. It is true that later chemical investigations contributed further important details, culminating in H. Fischer's (1a) *in vitro* synthesis of chlorophyll; and that studies on the state of chlorophyll in chloroplasts have furnished evidence that in its functional condition the pigment occurs combined with protein constituents. But Willstätter and Stoll's formulation of the basic structure of chlorophyll as a compound in which four pyrrol groups, each carrying its own characteristic side chains, are united into a porphin ring system, binding a central magnesium atom, has neither been challenged nor modified.

These fundamental contributions to our knowledge about the nature of chlorophyll also marked the beginning of a comprehensive attempt on the part of Willstätter and Stoll to analyze the photosynthetic process, and were followed, five years later, by the appearance of their “Investigations on carbon dioxide assimilation” (2). During the intervening years they had collected an impressive mass of experimental data, mostly dealing with the effects of various environmental conditions on the photosynthetic

reaction. Among the many important results of these studies, three stand out as being so fundamental that any treatise on photosynthesis should start with their reiteration. They can be paraphrased as follows: (a) There is no experimental evidence that the carotenoid pigments function in either a photochemical or non-photochemical ("dark") reaction during the photosynthetic process. (b) During photosynthesis under a large variety of experimental conditions the two chlorophyll components, a and b, do not appear to undergo any permanent or transitional changes. (c) The ratio of CO_2 assimilated to O_2 evolved is constant over a wide range of environmental conditions. Since its value is unity, the photosynthetic reaction can be described by the equation

Light



It is evident from this constant ratio that the transformation of CO_2 into assimilation products must proceed without the accumulation of intermediate compounds which differ in their elementary composition from carbohydrate.

Combined with the known chemical structure of the chlorophyll molecule, itself obviously involved as the functional light absorbing factor, these findings were used to develop an integrated concept of the mechanism of photosynthesis which soon became generally accepted as the basis for many future investigations.

The hypothesis evolved out of the foregoing studies starts with the assumption that the CO_2 is adsorbed on or bound by chlorophyll. The actual transformation of CO_2 during photosynthesis yields substances with the elementary composition CH_2O , without the detectable formation of any intermediate reaction products. Since formaldehyde is the only one-carbon compound with the required composition, it appeared logical to assume that CO_2 would first be converted to formaldehyde. The more complicated assimilation products were supposed to arise from this simple substance by condensation reactions.

Hence the photosynthetic process was considered as consisting essentially of a molecular rearrangement, proceeding under the influence of light, in which CO_2 or carbonic acid, combined with chlorophyll, is transformed into a chlorophyll-formaldehyde-peroxide complex. The latter was thought to be decomposed by special enzymes to the components, chlorophyll, formaldehyde, and oxygen, and by means of enzymatic condensation reactions the formaldehyde would finally be converted to carbohydrates, the conspicuous organic products of photosynthesis.

The production of formaldehyde by illuminated, chlorophyll-containing plant extracts occasionally had been reported by earlier experimenters. But the amounts involved had invariably been minute; and the more cautious interpreters of such results had pointed out that the formaldehyde could easily have been formed by photodecomposition of organic substances instead of by photosynthesis of CO_2 . Willstätter and Stoll now carried out experiments with pure chlorophyll and CO_2 . They could detect no formaldehyde nor oxygen production, and hence concluded that even for the first transformations in photosynthesis chlorophyll, CO_2 , and light are not sufficient; a more complicated system is required.

An opposite view has been adopted by Baly and his collaborators (3) on the basis of the alleged occurrence of a photosynthesis of carbohydrates from CO_2 under the influence of irradiated nickel carbonate. However, the numerous reinvestigations of this phenomenon by various competent workers have so completely failed to corroborate Baly's results that it seems entirely legitimate to disregard this work here.

For many years following the appearance of Willstätter and Stoll's publications, the theory of an intramolecular conversion of a chlorophyll- CO_2 complex under the influence of illumination remained the concept most generally adopted to account for the photosynthetic reaction. Subsequent to the experimental studies of Warburg and Negelein (53, 54) on the number of light quanta absorbed concomitantly with the assimilation of one molecule of CO_2 , the fundamental ideas were incorporated in more detailed *schemata*, which showed how the multiple quantum absorption could be harmonized with the concept that photosynthesis consisted of a series of at least six individual step reactions (e.g. Holluta, [4]). But in spite of several further attempts to adduce experimental evidence in its favor, neither the direct connection between CO_2 and chlorophyll, nor the production of formaldehyde as an important intermediate stage in the formation of carbohydrates from CO_2 , have ever been demonstrated unequivocally.

The possible existence of CO_2 -chlorophyll complexes, of vital significance to the Willstätter-Stoll hypothesis, has recently been investigated by Smith (5) in considerable detail. The results argue decisively against the occurrence of such compounds. Furthermore, these experiments are of importance because they show how earlier work, supposedly demonstrating such complex formation, can more reasonably be interpreted in line with Smith's own findings.

Much the same lot has befallen the experimental demonstration of formaldehyde production. Although Klein and Werner (6),

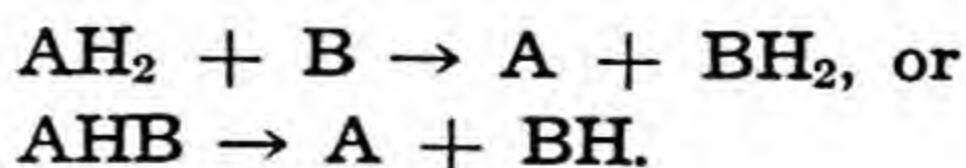
using the "trapping" technique in attempts to procure evidence for the intermediary formation of this substance during photosynthesis, obtained undeniably positive results, their studies have been subjected to much justifiable criticism (7). And from the most recent investigations on the position of formaldehyde in the general framework of the chemical activities of green plants, (8) the conclusion has been drawn that externally supplied formaldehyde cannot be used for carbohydrate synthesis. This obviously eliminates formaldehyde as an important intermediate product in photosynthesis.

From the foregoing paragraphs it is thus evident that the original Willstätter-Stoll hypothesis lacks a satisfactory experimental foundation. The recently reported production of equimolar quantities of formaldehyde and oxygen by an illuminated paste of chlorophyll dissolved in lanolin (9) seems to invalidate this statement. But unqualified acceptance of so spectacular a claim, on the basis of the accessible abstracts, is very difficult indeed. Moreover, other attacks have meanwhile been exploited, and these have resulted in a rather different outlook on the problem of photosynthesis. The introduction of the "comparative biochemical" approach has here played an important part, and it is with this point of view that the present essay is concerned.

THE MAIN FACTORS INVOLVED IN THE COMPARATIVE BIOCHEMICAL APPROACH

THE GENERALIZED FORMULATION OF PHOTOSYNTHESIS

During the 1920's it had become evident that the typical catabolic processes of microorganisms, of plants, and of animals exhibit a striking mutual resemblance, perhaps not so much in their final outcome as in the similarity of many of the step reactions into which they can be resolved. This recognition, together with the fundamentally important concept that each of these steps constitutes an extremely simple type of reaction, gave rise to the development of a working hypothesis which Kluyver (10), himself so largely responsible for its enunciation and development into an invaluable tool, has aptly designated as "comparative biochemistry." It postulates as a fruitful central idea that all metabolic activities are intrinsically similar, and that each consists of a more or less extended series of inter- or intramolecular hydrogenation-dehydrogenation reactions. The individual steps can thus be represented by a general equation of the type



Depending upon the chemical nature of the participants in a reaction, some variations may be encountered, but for a given system of components the final outcome is limited to very few possibilities.

This is not the place to go into an elaborate exposition of the impressive experimental foundation for the comparative biochemical outlook. Adequate accounts for this purpose are available in a number of publications (10-14). Suffice it here to state that this approach has achieved a far-reaching integration of knowledge in the field of biochemistry, and succeeded in correlating the multitudinous manifestations of the chemical activities of living organisms. It has aided in analyzing hitherto obscure processes by comparing them with better known ones in which the same substrates participate. The elucidation of the bacterial methane fermentation provides a striking example of the successful application of such a comparison. Oxidation of the various substrates which can serve for a methane fermentation had been known to occur with oxygen, nitrate, or sulfate as ultimate hydrogen acceptors, the latter yielding water, ammonia, or hydrogen sulfide as the corresponding reduction products. Viewed in this light, the methane fermentation could be regarded as a fundamentally similar process in which the substrate oxidation is coupled with a complete reduction of CO_2 to methane. The validity of this interpretation of the methane fermentation as a carbonate reduction has been demonstrated by the skillful experiments of Barker (15-17).

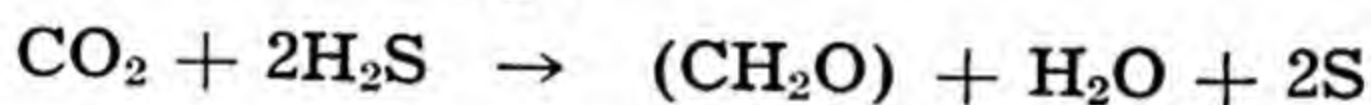
An application of the principles of comparative biochemistry to photosynthesis might therefore be expected to contribute to a better understanding of this biochemical phenomenon.

As long as green plant photosynthesis was the only process in which a conversion of CO_2 into organic substances was known to occur, it was obviously impossible to treat this problem as one to be dealt with in the light of comparative biochemistry. But the situation was radically changed when the studies on the metabolism of the green and purple sulfur bacteria led to the conviction that these organisms, too, carry out a photosynthetic reaction which, however, differs in some major respects from that characteristic of green plants. The experimental evidence for the photosynthetic mode of life of the above-mentioned microorganisms can be summarized in the following statements (18-21): (a) They can grow in a strictly inorganic medium, and hence must be able to synthesize their cell material from CO_2 which is then the only available carbon source. (b) Such growth is limited to conditions in which the cultures are appropriately illuminated. These observations

conclusively demonstrate the photosynthetic nature of the metabolism of the green and purple bacteria. Nevertheless, the process is not identical with photosynthesis as carried out by green plants. This appears clearly from the equally well established facts that, in the case of the bacteria: (c) Development is strictly dependent on the presence of hydrogen sulfide in the culture medium. (d) In no case has oxygen evolution been observed in cultures of these organisms, and this in spite of applying such extremely sensitive methods for oxygen detection as, e.g., the one in which luminous bacteria are used as indicators.

The dependence of growth on the supply of hydrogen sulfide can be expressed as a quantitative relationship. The green sulfur bacteria continue to develop as long as hydrogen sulfide is present; the disappearance of the latter coincides with the accumulation of elementary sulfur. And as soon as the last traces of sulfide have been so converted, growth ceases. Quantitative chemical analyses have shown that the changes in the medium agree, as a first approximation, with the equation:

Light



in which the symbol (CH_2O) is used to denote the organic matter produced in the form of bacterial cells.

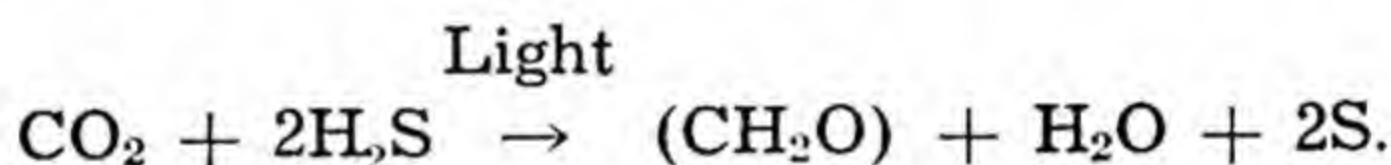
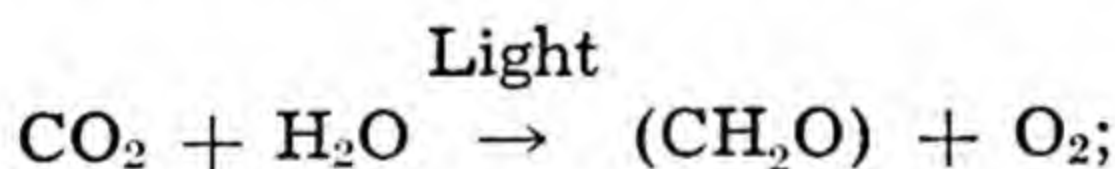
Now the conversion of hydrogen sulfide to sulfur under the influence of bacterial cells had been known since 1887, when Winogradsky (22, 23) published his epoch-making studies on the sulfur bacteria. This process was at that time interpreted as a physiological oxidation process, equivalent to the much more common oxidations of organic substances by living organisms. A deeper penetration into the mechanism of biological oxidations was subsequently made possible by Wieland's important generalization (24) to the effect that such processes should be considered primarily as dehydrogenations of the organic (or oxidizable) substrate with oxygen fulfilling the function of ultimate hydrogen acceptor. Application of this principle to Winogradsky's sulfur bacteria would thus mean that their chief metabolic activity consists in a dehydrogenation of hydrogen sulfide to sulfur with a transfer of hydrogen to oxygen.

Evidently, the green sulfur bacteria bring about the same conversion of sulfide to sulfur. However, they can carry out this oxidation in the complete absence of oxygen. Here, then, the advantages of a comparative-biochemical approach become evident, for if the sulfur production is to be considered as a dehydrogenation

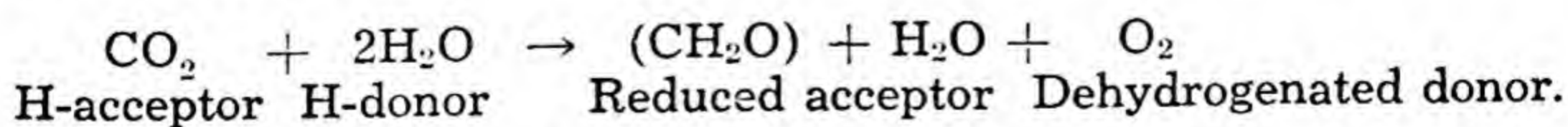
reaction of the sulfide, it is obvious that it must proceed with a hydrogen acceptor other than oxygen. A consideration of the constituents of the medium reveals that only CO_2 would be available as such. Furthermore, it is known that CO_2 actually becomes converted into organic cell constituents, a transformation which corresponds to a reduction. Consequently, the equation representing the metabolism of the green bacteria can be paraphrased by the statement that hydrogen sulfide is dehydrogenated to sulfur, with CO_2 functioning as the final hydrogen acceptor, resulting in its reduction to cell material.

Up to this point in the discussion the comparative biochemical approach to the metabolism of the green bacteria has been restricted to a comparison of their activities with those of other sulfur bacteria. It has already been emphasized, however, that the former must be designated as photosynthetic because of its complete dependence upon proper illumination. Consequently a different comparison, this time with the photosynthetic process of green plants, is also justified.

The reaction equations for the photosynthesis of green plants and of green bacteria actually reveal a close similarity:



Since the previous argument has shown that the latter process can be regarded as a dehydrogenation of hydrogen sulfide with CO_2 as ultimate hydrogen acceptor, it becomes tempting indeed to apply the same viewpoint to the former. In so doing the ordinary photosynthetic process emerges as a reaction in which CO_2 is reduced by hydrogen derived from water rather than from a readily oxidizable substrate. Oxygen evolution is then the necessary consequence of the dehydrogenation process, and entirely comparable with sulfur formation from sulfide by the green bacteria. The similarity becomes even more explicit if, in accordance with this concept, the equation is rewritten in the following form:



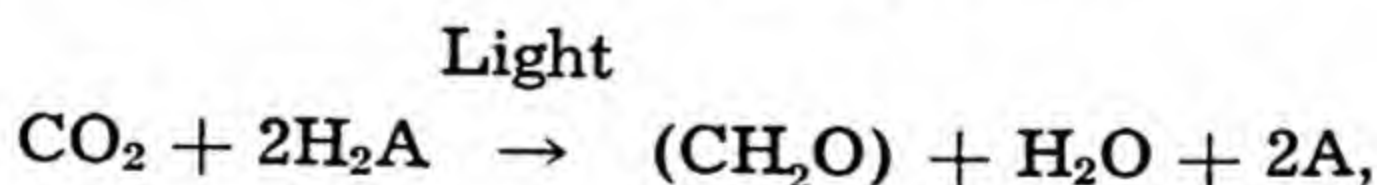
On the basis of this formulation green plant photosynthesis should no longer be considered as involving an intramolecular rearrangement of CO_2 or carbonic acid, combined with chlorophyll,

to formaldehyde peroxide; it becomes one of the numerous examples of a typical biochemical reaction, a coupled hydrogenation-dehydrogenation.

The use of hydrogen sulfide as hydrogen donor in photosynthesis is not restricted to the green bacteria; the purple sulfur bacteria are also capable of effecting a CO_2 reduction in light in the presence of sulfide. In this instance, however, oxidation of the sulfide proceeds beyond the stage of elementary sulfur, and goes to sulfate. As might be anticipated, this complete oxidation is accompanied by a photoreduction of a correspondingly greater amount of CO_2 .

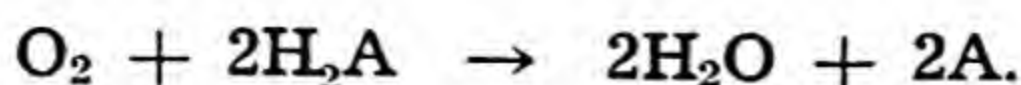
Again, a comparison with the activities of the non-photosynthetic sulfur-oxidizing bacteria is instructive. There exist a number of colorless microorganisms that can accomplish a similar oxidation process. In these cases the oxidation is dependent upon the presence of hydrogen acceptors other than CO_2 , such as oxygen or nitrate. It is reasonable to suppose that the conversion of sulfide to sulfate passes through a number of intermediate oxidation stages, and this assumption is supported by the fact that the colorless sulfur bacteria which have been investigated in this respect have been found capable of oxidizing such compounds as sulfur, sulfite, and thiosulfate. It could be expected, therefore, that the purple sulfur bacteria would also be able to utilize these incompletely oxidized substances, and, in illuminated cultures, transform them into sulfate with a simultaneous reduction of CO_2 . Pertinent experiments have served to verify this (19).

Hence the development of a concept of photosynthesis along the lines of comparative biochemistry resulted in the characterization of this process as a photochemical CO_2 reduction involving the participation of any one of a number of different reducing agents as hydrogen donors. A general formulation of the various photosyntheses thus takes the form of the equation:



wherein H_2A represents any one of a variety of oxidizable compounds, and A their corresponding oxidation (dehydrogenation) products.

This, in turn, made it possible to speculate still further by comparing photosynthesis with diverse biological oxidations, since the latter can be comprehensively rendered by a strikingly similar equation (10):

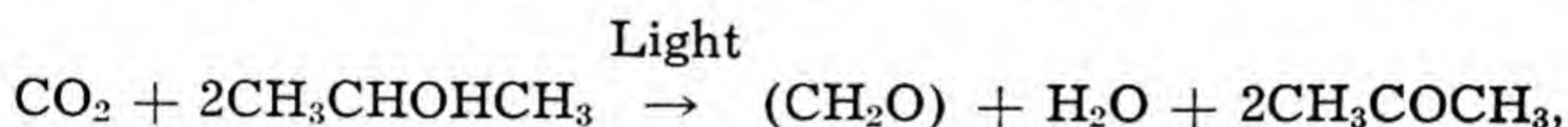


Especially the knowledge that virtually any oxidizable substance can be oxidized by some living organism suggested that photosynthetic reactions might exist in which the hydrogen donor is represented by some substance other than either water or a reduced sulfur compound. And the experiments of the past twelve years have amply confirmed this; photosyntheses are now known in which the reducing agent is a reduced selenium compound (25), molecular hydrogen (26, 27), or any one of a number of simple organic substances (27-30).

There are some complications connected with the last-mentioned group which make a more detailed treatment of such processes desirable. In most biological oxidations the decomposition of organic materials yields, as end products, CO_2 and water. If this were the fate of organic substrates utilized in the photosynthetic reactions carried out by the purple bacteria in organic media, the CO_2 production resulting from the substrate oxidation could easily obscure the quantitative detection of CO_2 utilization (reduction) during photosynthesis. It is readily conceivable that chemical determinations of the CO_2 balance would show neither the production nor the disappearance of this substance; such would be the case whenever the composition of the substrate, with respect to its state of oxidation, was the same as that of the final photosynthate, i.e., the bacterial cells. The over-all equation representing this type of decomposition might thus be interpreted to mean that organic substances are directly converted into cell material under the influence of light, a suggestion actually put forward by Molisch (31). Nevertheless, the transformation of substrate into cell material could well have been indirect, and might actually have involved a complete dehydrogenation of the organic substrate, with the production of the theoretical amount of CO_2 , and accompanied by the photoreduction of an identical quantity of the latter substance. CO_2 utilization would be observable only with substrates that are more reduced than the cell material. The experimental results of Gaffron (27, 28) and of Muller (29) can be adequately accounted for on this basis. However, it must be clearly realized that such data cannot be used as positive evidence for the above postulated role of organic substances as hydrogen donors in the photosynthetic metabolism of the purple bacteria. It is merely the concatenation of arguments derived from comparative biochemical considerations that makes such an interpretation consistent and plausible (20).

Unambiguous experimental support for the view that organic substances serve as hydrogen donors in bacterial photosyntheses was supplied by Foster (32) in 1940, and once again the success of these investigations must be credited to the comparative bio-

chemical approach. A clear-cut demonstration that an organic substrate can serve in this manner would be possible if compounds could be found which are oxidized without the evolution of CO_2 , and preferably, without a change of their carbon skeleton. It is especially the oxidation of alcohols by a number of colorless microorganisms which has furnished many striking examples of such incomplete oxidations. In this manner Foster was led to investigate the photosynthetic decomposition of primary and secondary alcohols by purple bacteria (32, 33). The experiments showed that the primary alcohols are completely converted into cell material. But various secondary alcohols are quantitatively oxidized to the corresponding ketones. In illuminated cultures of purple bacteria, under anaerobic conditions, this oxidation is accomplished with the concomitant conversion of a corresponding amount of CO_2 into cell material. By establishing that the utilization of iso-propanol, for example, can be accurately represented by the equation:



Foster provided the first incontrovertible evidence that an organic substance can function exclusively as hydrogen donor in a photosynthetic process.

Although the chain of circumstantial evidence for the postulated role of organic compounds in bacterial photosynthesis was hereby considerably strengthened, the extension of this concept to substances that are utilized without leaving easily recognizable decomposition products behind remained only a probability. Obviously, the direct experimental demonstration of an actual CO_2 assimilation by purple bacteria acting upon organic substrates which give rise to CO_2 production had to await the development of isotope techniques. The availability of CO_2 containing the radioactive carbon isotope, C^{14} , offered a possible means of procuring more direct evidence. In as yet unpublished investigations, carried out in collaboration with Dr. H. Albert Barker, this missing link has been supplied.

Cultures of non-sulfur purple bacteria were grown anaerobically with proper illumination. The culture media contained the necessary minerals and vitamins, as well as acetate, propionate, normal or isobutyrate, lactate, or succinate as the specific oxidizable substrate. CO_2 was provided as radioactive bicarbonate, prepared from $\text{BaC}^{14}\text{O}_3$. Analysis of such cultures has shown that CO_2 is used in the synthesis of cell material, since the bacterial cells were found to contain considerable quantities of C^{14} in the form of organic substances. Similar results were obtained in short-term

experiments with dense cell suspensions which were allowed to photosynthesize in the presence of the above-mentioned organic substrates and $C^{14}O_2$, under conditions where cell multiplication was excluded.

A quantitative evaluation of the data collected in these experiments leads, however, to the conclusion that the organic substrates do not serve exclusively as hydrogen donors for a photochemical CO_2 reduction; the amount of C^{14} in the cells falls short of the theoretical requirements of such a mechanism. The evidence indicates that a far from negligible proportion of the substrates is used more or less directly for conversion into cellular constituents. This phase of the problem will be amplified in a later section; suffice it to state here that such results had been anticipated on the basis of comparative biochemical considerations.

In spite of this quantitative discrepancy it appears justifiable to conclude that the generalized concept of photosynthesis as a photochemical CO_2 reduction which requires specific hydrogen donors has been firmly established by the experimental evidence discussed in the preceding pages. (For a discussion of the inadequacy of alternative hypotheses concerning purple bacteria photosyntheses see, 34, pp. 282-86.)

THE PRODUCTION OF OXYGEN IN GREEN PLANT PHOTOSYNTHESIS

The above formulation implies that photosynthesis accompanied by oxygen evolution, the outstanding characteristic of this process as carried out by green plants, results from the use of water as the specific hydrogen donor. This does not mean, however, that other donors cannot be used by these organisms. And, in fact, Gaffron has shown that one may encounter or induce conditions under which certain green algae photosynthesize without producing oxygen. The assimilation of CO_2 is then linked with the oxidation of external hydrogen donors other than water, *in casu* molecular hydrogen (35, 36). This particular reaction is the only case of green plant photosynthesis that has so far been demonstrated to proceed without oxygen evolution and with the aid of a special reducing agent. It is, however, not unlikely that many other cases of a similar nature exist, and that, for example, various organic compounds may also participate under special conditions in the photosynthetic process of green plants, just as they do in purple bacteria photosyntheses. Possibly such conditions are often realized locally or temporarily in the cells of photosynthesizing plants, with the consequence that part of the photosynthetic mechanism would then involve hydrogen donors other than water and represent photosynthesis without the production of oxygen. For lack of available evidence this theme will not be further discussed.

It does, however, seem pertinent at this point to examine another aspect of the photosynthesis problem from the point of view of comparative biochemistry. This is the evolution of oxygen itself. According to the generalized concept of photosynthesis, oxygen production is the consequence of the use of H_2O as the specific hydrogen donor for CO_2 reduction. It follows that the oxygen liberated in green plant photosynthesis must be derived exclusively from water, and not in whole or in part from CO_2 . When oxygen isotopes became available for research this consequence could be tested experimentally.

The first investigations of this kind were carried out by Ruben *et al.* (37), and by Vinogradov and Teis (38), who determined the composition of oxygen evolved during illumination of green plants in systems in which either the CO_2 or the water contained an abnormally high proportion of the heavy oxygen isotope, O^{18} . It was found that the composition of the gaseous oxygen corresponded to that of the H_2O -oxygen, and not to that of the CO_2 . Hence the conclusion was drawn that the results supported the postulated origin of photosynthetic oxygen.

But this interpretation has been subjected to the criticism that exchange reactions between the oxygen in water and in CO_2 may have proceeded inside the plant cells at rates considerably different from those determined for the external medium (39). It is, therefore, fortunate that similar isotope experiments have been conducted under natural equilibrium conditions (40). The results of these studies, not open to the above criticism, likewise indicate that the source of the evolved oxygen is water, and not CO_2 .

Notwithstanding these apparently satisfactory results, all is not well. Yosida *et al.* (41) have described similar experiments with submerged water plants, carried out under equilibrium conditions, and concluded from the isotopic composition of the oxygen produced that a fraction, estimated at about 33 per cent, is derived from CO_2 . Moreover, Kamen and Barker (39) have pointed out that the isotopic composition of the atmospheric oxygen, generally and on good grounds supposed to be derived from photosynthesis, does not entirely correspond to that of the water on earth. The observed discrepancy cannot be explained by a preferential utilization of O^{16} by soil microorganisms (42). It is, of course, possible that exchange reactions which have not hitherto been considered are responsible for the noted lack of agreement. It must also be kept in mind that the experiments to date have been restricted to a very small number of representatives of the plant kingdom.

However this may be, it must be conceded that a fully convincing and direct demonstration of the source of photosynthetic oxygen is still lacking. This makes it necessary to discuss some

phenomena which permit a decision along the indirect lines of reasoning supplied by comparative biochemical arguments.

In 1920 Warburg and Negelein (43) discovered that suspensions of the green alga, *Chlorella pyrenoidosa*, can reduce nitric acid to ammonia. In darkness this reduction is accompanied by an oxidation of organic cell materials, and consequently by CO_2 production. The process is, in most respects, similar to the nitrate reduction accomplished by a variety of bacteria when they oxidize certain organic or inorganic compounds in nitrate solutions under anaerobic conditions. At present it can be reasonably interpreted as a form of oxidative metabolism in which molecular oxygen as the ultimate hydrogen acceptor is replaced by nitrate.

Now it is a well-known fact that most green plants can manufacture their protein constituents from nitrate-nitrogen, a process which obviously implies a reduction to the amino stage. Therefore, the occurrence in plants of a nitrate reduction similar to that encountered in various bacteria could have been anticipated.

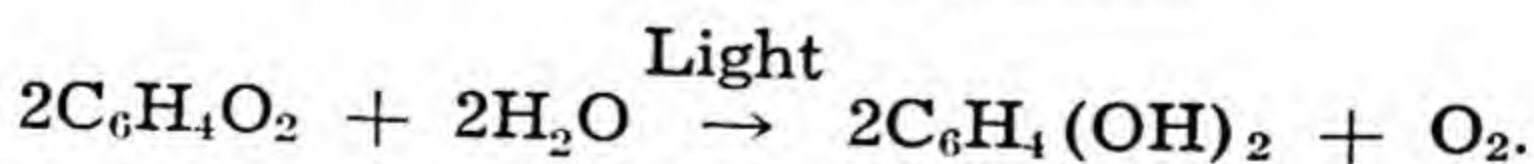
On the other hand, the outcome of Warburg and Negelein's further experiments, dealing with the influence of light on the reduction of nitrate, was entirely unexpected. The results showed that in illuminated suspensions of *Chlorella* nitrate reduction proceeds with the simultaneous evolution of oxygen instead of CO_2 . At first sight it would appear that this phenomenon could be simply interpreted as due to the superposition of a normal photosynthetic reaction upon nitrate reduction, since the production of CO_2 in the latter process would furnish the plants with one missing ingredient necessary for typical photosynthesis. But the order of magnitude of the oxygen liberation is very much greater than that which could be accounted for on this basis; the rate of CO_2 formation during nitrate reduction in darkness, upon which the photosynthetic process would have to depend, is far too low to explain the observed rate of oxygen production. It is, of course, possible to reconcile these discordant facts by assuming that the rate of nitrate reduction—and consequently of CO_2 formation—is considerably increased by illumination, a supposition advanced by Warburg and Negelein at that time. Nevertheless, an alternative explanation suggests itself, which appears far more plausible from the viewpoint of comparative biochemistry. It is based on the possibility that a *Chlorella* suspension, illuminated in the absence of CO_2 , might carry out a modified photosynthetic reaction. Obviously, CO_2 need not be the ultimate acceptor for hydrogen released by a photodecomposition of water. It is quite conceivable that nitric acid could function in this capacity. If this were the case, the oxygen production observed by Warburg and Negelein would be due to the occurrence of the above postulated photolysis of water in the

presence of a satisfactory hydrogen acceptor other than CO_2 . The rate of nitrate reduction and of oxygen evolution would then no longer be dependent upon the rate of oxidation of organic cell materials, but would be determined by the rate at which water can be dehydrogenated by the plants in the light.

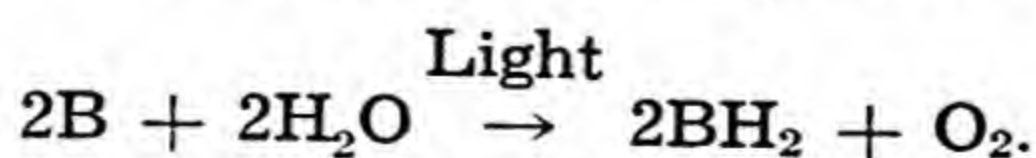
This latter interpretation gains considerably in probability by virtue of the fact that other instances of green plant photosynthesis are known in which oxygen is produced without an accompanying CO_2 reduction. One such case has been described by Fan *et al.* (44) who reported that a *Chlorella* suspension, illuminated in a CO_2 -free system, may produce oxygen with the simultaneous reduction of benzaldehyde to benzyl alcohol. Other examples of the same type have been furnished by experiments with isolated chloroplasts.

In 1937 R. Hill (45) first announced that isolated chloroplasts can, upon illumination, produce oxygen in the presence of ferric oxalate. It also became apparent that CO_2 plays no role in this reaction. Consequently it was possible to interpret the observed phenomena as a photodecomposition of water with the simultaneous reduction of ferric oxalate instead of CO_2 under the influence of the chloroplasts (46). Rabinowitch (47, p. 66) succinctly stated that it seems very probable that "broken or dried chloroplasts retain an important part of their normal photocatalytic capacity—they can still produce oxygen from water in light. However, they are not able any more to transfer hydrogen to CO_2 as acceptor, and thus cannot synthesize organic matter."

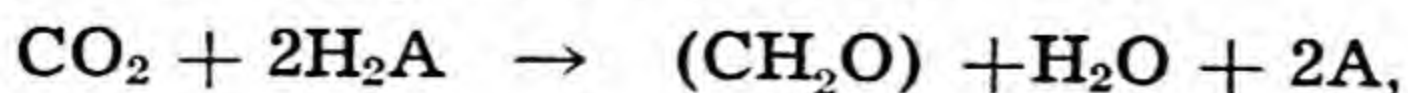
French and co-workers (48–51) have followed up Hill's experiments, and recently demonstrated that various dyes can be similarly reduced with a concomitant evolution of oxygen. And Warburg and Lütgens (52) have added quinone to the list of agents now known to permit the production of oxygen by illuminated chloroplasts, whereby the quinone is converted to hydroquinone in approximate accordance with the equation:



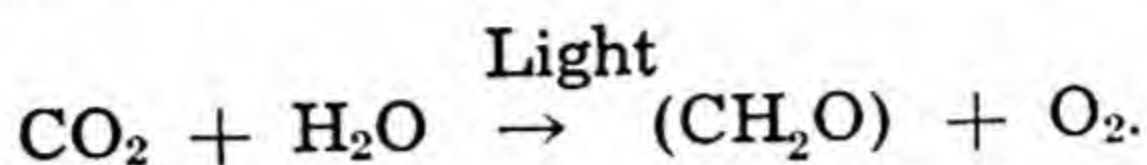
All of the above-mentioned cases in which oxygen production in light is not accompanied by an equivalent reduction of CO_2 can easily be understood as special examples of a process which, in its most generalized form, can be expressed by the equation:



This equation, a counterpart of the earlier one:

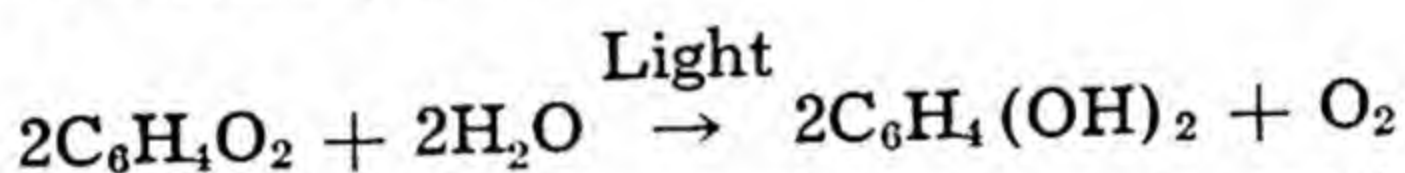


represents that aspect of photosynthesis in which a photochemical dehydrogenation of water is accomplished with the simultaneous reduction of any one of a number of possible hydrogen acceptors, B. And only when this acceptor is CO_2 does the equation revert to the one originally used to describe green plant photosynthesis:

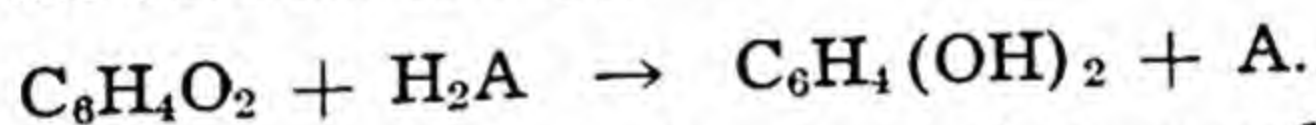


The instances in which quinone or benzaldehyde fulfill the role of hydrogen acceptors are especially instructive because the recovery of these compounds as hydroquinone and benzyl alcohol clearly indicates their function. Since CO_2 is not at all involved in the process, the corollary is that the evolved oxygen must, in its entirety, have originated from the water.

That quinone can be used so effectively to elucidate the source of photosynthetic oxygen is of particular interest for the following reason. The reaction equation



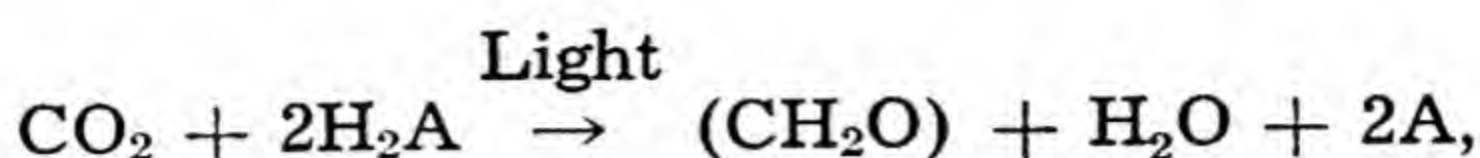
bears a striking resemblance to the one representing the now classical reduction of quinone during the oxidation of various substances, collectively designated as H_2A , by a large number of organisms in the absence of oxygen:



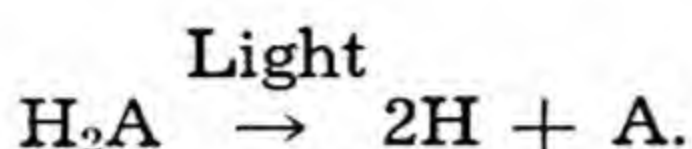
It was this last-mentioned type of process that served more or less as the starting point for Wieland's development of the concept that biological oxidations are in essence hydrogen transfer reactions, and that oxygen is only one of a number of possible hydrogen acceptors in such reactions (24). It is the corresponding experiments of Warburg and Lütgens on photosynthesis by chloroplasts in the presence of quinone, described above, that can now be invoked to support the thesis that CO_2 is only one of a number of possible hydrogen acceptors in the photosynthetic process. It further follows that the fundamental nature of photosynthesis, as far as the photochemical reaction is concerned, represents a dehydrogenation of water under the influence of light absorbed by special pigment systems ("Photocatalysts").

THE PHOTOCHEMICAL REACTION IN BACTERIAL PHOTOSYNTHESSES

The concept developed in the preceding section, viz., that the specifically photochemical process of green plant photosynthesis is a photolysis of water, has obvious implications for an interpretation of the mechanism of the bacterial photosyntheses. On the basis of the general formulation:



it might be argued that the substitution of H_2S , H_2 , or some organic compound for H_2O as hydrogen donor should mean that it is those substances which are here photochemically decomposed, and that the photochemical reaction in bacterial photosyntheses could be represented by



In an earlier publication (34) I have, however, pointed out that such a conclusion leads to very undesirable consequences. The most serious difficulty is that every substrate which the photosynthetic bacteria can use as " H_2A " in a photosynthetic reaction can also be oxidized in darkness by a variety of organisms, and that even the photosynthetic bacteria themselves can do so provided that oxygen be present. This behavior can be demonstrated in a most striking manner by the following experiment.

Equal quantities of a suspension of purple bacteria in a dilute NaHCO_3 -solution are prepared, and each is supplied with the same hydrogen donor. One aliquot is illuminated in an atmosphere of nitrogen containing 5 per cent CO_2 ; the second is kept in darkness, but with the same gas phase as the first; the third, also in darkness, is exposed to air with 5 per cent CO_2 . If the light intensity used for illuminating the first suspension is sufficiently high to insure photochemical saturation, the temperature being the same for all three, it is found that the rate of oxidation of the substrate is identical in the first and third of these cultures; in the second the amount of substrate remains unchanged.

Experiments of this sort show conclusively that the dehydrogenation proper of the various hydrogen donors can be accomplished by enzyme systems that function in the absence of light. Illumination is required only when CO_2 is the sole available final hydrogen acceptor.

It also follows that light and oxygen are interchangeable in the metabolism of these bacteria. This peculiarity would be readily understandable by considering the photosynthetic process as one in which oxygen is liberated from CO_2 in light. Yet we have seen that this interpretation is untenable, because suspensions of photosynthetic bacteria fail to produce oxygen when illuminated, even in the absence of a reducing substrate.

But molecular oxygen is not the exclusive substance which, in the dark, permits the purple bacteria to oxidize a particular substrate. Gaffron (28) has shown that at least one strain is capable

of such an oxidation with nitrate as acceptor, and many unpublished experiments have convinced me that all purple bacteria can oxidize appropriate substrates in the dark under anaerobic conditions when supplied with methylene blue, quinone, or other commonly used hydrogen acceptors. In order to account for the fact that these organisms can grow either when illuminated under anaerobic conditions or in darkness in the presence of air it is, therefore, not necessary to assume that illumination yields oxygen; the formation of some other hydrogen acceptor would explain the situation equally well.

Measurements of the redox potentials in suspensions of purple sulfur bacteria by Roelofsen (26) provide a strong indication that such substances are actually formed, because the potential of an anaerobic suspension of these organisms increases rapidly and considerably upon exposure to light.

I have recently tried to demonstrate in a different way the presence of hydrogen acceptors in anaerobic suspensions of purple bacteria that had been illuminated for some time in the presence of CO_2 without the addition of a hydrogen donor. This was done by introducing a readily oxidizable substrate at the moment at which the cultures were deprived of light, and determining whether the latter was subsequently decomposed in darkness. The results to date have been completely negative. Prolonged preillumination does not alter the outcome of the experiments; hence a progressive accumulation of oxidizing substances does not take place. It must therefore be concluded that the total quantity of hydrogen acceptors formed in the light is at best extremely small, and escapes detection by the method used.

Nevertheless, the assumption that a reducible system is formed in light is an unavoidable consequence of the concept that dehydrogenation of the specific substrates by purple bacteria takes place by means of dark reactions. And the evidence presented above in favor of this view seems to me sufficiently compelling to reject the postulate of a contrary hypothesis, *viz.*, the direct participation of the oxidizable substrate in a photochemical reaction. The photochemical production of hydrogen acceptors must then be relegated to a process involving either H_2O or CO_2 , the only remaining components of the environment.

There are good reasons for considering the mechanism by which the assimilation and reduction of CO_2 is accomplished as composed exclusively of dark reactions, a proposition more fully developed in the next sections. This would restrict the possibility for the photochemical process in bacterial photosyntheses to a reaction involving H_2O .

Nor is this conclusion difficult to accept. Application of the principles of comparative biochemistry strongly suggests that in photosynthetic bacteria as in green plants the photochemical reaction proper would be similar, *i.e.*, would be a photolysis of water. The situation presented by the former organisms requires, however, that here the photolysis does not yield molecular oxygen.

Oxygen evolution in green plant photosynthesis can best be interpreted as resulting from subsequent transformations of a photolytic product via a peroxide. The assumption that in the bacterial counterpart no such peroxide is formed would go far in accounting for the differences in the two main types of photosynthesis. Elsewhere (34, p. 322-24) I have postulated that a photochemical act would result in the formation of an oxidized enzyme system, the photochemical reaction being dependent upon the presence of the enzyme in a reduced form. In green plants the oxidized system can be reconverted into the reduced state by the elimination of molecular oxygen. The rate of photosynthesis at high light intensity might then become limited by the velocity at which the regeneration of the reduced enzyme occurs. In photosynthetic bacteria, however, a similar regeneration would have to be achieved by a reduction of the oxidized form with the aid of external hydrogen donors; in the absence of the latter this particular enzyme system would soon exist only in its oxidized state and thus prevent further photosynthesis. This hypothesis would explain the strict dependence of bacterial photosyntheses on the availability of special hydrogen donors whose dehydrogenation is accomplished by dark reactions. It would also account for the absence of oxygen production and for the observed increase in oxidation-reduction potential in illuminated cultures of purple bacteria. And finally, the failure to demonstrate the accumulation of hydrogen acceptors in such cultures by the experiments described above becomes understandable, because the results could have been positive only if a fairly large amount of such compounds had been formed. This can hardly be expected if the hydrogen acceptors represented an enzyme system in its oxidized form.

It may thus be concluded that the evidence so far available is consistent with a characterization of the photochemical reaction in bacterial photosyntheses as a photolysis of H_2O .

THE ASSIMILATION OF CO_2 IN PHOTOSYNTHESIS AS A DARK REACTION

The conversion of CO_2 into organic substances requires a supply of energy. In photosynthetic processes this requirement is met by the absorption of radiant energy. But this does not imply that the complex mechanism by which organic matter is produced from

CO₂ during photosynthesis must be considered as a series of exclusively photochemical reactions.

In fact, it has been known since Blackman investigated the influence of various environmental factors on the photosynthetic rate that the assimilation of CO₂ by green plants is the net result of a composite of both photochemical and non-photochemical ("dark") reactions. The bacterial photosyntheses, likewise are processes comprising both types of reactions (cf. 34). It thus follows that an important aspect of the analysis of photosynthesis consists in clearly designating the general nature of the photochemical and of the dark reactions.

In the preceding sections it has been shown that certain experimental results lead to the conclusion that in all photosyntheses the only photochemical reaction may be regarded as a photodecomposition of water. Acceptance of this view necessarily relegates the conversion of CO₂ into organic matter to the category of dark reactions. The present section will deal with two additional lines of evidence pointing in the same direction. The arguments are drawn from kinetic studies of photosynthetic processes on the one hand, and from considerations of the role of CO₂ in the metabolism of some photosynthetic and non-photosynthetic organisms on the other.

Kinetics

In green plants the photosynthetic reaction requires, for the reduction of CO₂ to carbohydrate, a theoretical minimum of four quanta per molecule of CO₂. The relationship between the number of quanta absorbed and molecules of CO₂ assimilated was first determined in the classical experiments of Warburg and Negelein (53, 54). From these it appeared that photosynthesis could actually proceed with this minimum number of quanta. Recent investigations (55-63) have consistently yielded values that are about two or three times as high; only Warburg (64) has maintained the earlier established value of four.

It is true that an accurate knowledge of the minimum number of quanta is essential for a critical appraisal of certain proposed mechanisms of photosynthesis. However, this is not the case for the present argument. What matters here is the recognition that photosynthesis is a process in which several quanta must be absorbed to permit the reduction of a single CO₂ molecule.

Under otherwise constant conditions the rate of photosynthesis is directly proportional to the incident light energy over a certain range. Such dependence implies that the photochemical reaction proper is a first-order reaction; a quantum absorbed evidently

induces a process which results in CO_2 assimilation. This situation is incompatible with the assumption that CO_2 participates directly in the photochemical part of the process, as the following considerations will show.

Current concepts of photochemical reactions hold that a light quantum, absorbed by some molecule, causes an "activation" of the latter. In this new state it can undergo or initiate reactions which it cannot provoke in its inactive state. A first-order photochemical reaction is one wherein the requisite activation is brought about by the absorption of a single quantum.

Many cases are on record showing that light absorbed by chlorophyll alone is effective in producing photosynthesis. Hence the photochemical reaction should here be one between an activated chlorophyll molecule and a molecule of another type. Assuming that the latter were CO_2 , an insurmountable difficulty arises.

As has been mentioned, the conversion of CO_2 to "carbohydrate" and oxygen is impossible without the absorption of at least four quanta of radiant energy per molecule of CO_2 . Because the photochemical process is a first-order reaction, any hypothesis concerning a mechanism which involves the simultaneous absorption of several quanta by one and the same chlorophyll molecule, a mechanism which would have the characteristics of a higher-order reaction, must be rejected. It is therefore evident that the quanta must be absorbed consecutively by the same pigment molecule, or that quanta absorbed by different chlorophyll molecules can produce an effect on the same CO_2 molecule.

The first of these possibilities implies a mechanism whereby a CO_2 molecule undergoes a stepwise transformation into the products of photosynthesis by means of a series of photochemical reactions effected by a single chlorophyll molecule. Each successive absorption of a quantum would thus initiate a single step. But such a mechanism is irreconcilable with the immediate onset of photosynthesis at low light intensity. Kohn (68) has calculated that under conditions realized in some experiments the consecutive absorption of four quanta by the same chlorophyll molecule would occur only once in several years; photosynthesis was, however, observed immediately upon illumination.

The second possibility has received direct experimental support from the ingenious studies of Emerson and Arnold (69) and of Arnold and Kohn (70). These investigations have shown that the energy of randomly absorbed quanta can be effectively summated. The results imply that, if four quanta were needed for the production of a molecule of oxygen from CO_2 , oxygen evolution would occur when any four of a mass of about 2,000 chlorophyll molecules each absorb one quantum; or, if ten quanta were required, their

absorption by any ten chlorophyll molecules in 5,000 would produce photosynthesis. It seems impossible to account for such cooperation of spatially separated chlorophyll molecules by assuming that a photochemical reaction would occur at one activated pigment molecule where a molecule of CO_2 would undergo some small change; that the resultant partial transformation product would react elsewhere with another activated chlorophyll molecule; and so on, until all the successive conversions—at least four, and quite possibly more—had been accomplished. In the first place such an assumption would call for the inclusion of several different photochemical reactions, each under the influence of the same activated substance. This is highly improbable. In the second place it must be remembered that relatively high CO_2 concentrations are generally employed in photosynthesis studies. Under these conditions the chances overwhelmingly favor a reaction of an activated chlorophyll with a CO_2 molecule rather than with a partial conversion product. As a consequence the major event to be observed would be the partial transformation of CO_2 , and at low light intensity very little, if any, normal photosynthesis could occur. This is manifestly contrary to fact.

Although the above considerations are based on results obtained in studies of green plant photosynthesis, kinetic investigations of bacterial photosyntheses have shown that here the same situation is encountered (34). It is, therefore, evident that photosynthesis cannot be readily explained as a process in which CO_2 participates directly in a photochemical reaction.

This deduction is entirely in line with the conclusion reached in a previous section, *viz.*, that the photochemical reaction in photosynthesis involves water instead of CO_2 as the substance reacting with an activated chlorophyll molecule. The idea of a CO_2 assimilation exclusively by means of dark reactions is supported by the following evidence.

CO_2 Assimilation in Darkness

It has long been known that there exist microorganisms capable of manufacturing all their cell constituents in darkness from CO_2 as the only carbon source. These "chemosynthetic" or "chemoautotrophic" bacteria meet the energy requirements for CO_2 assimilation by the oxidation of inorganic substances. Outstanding examples are the bacteria that oxidize hydrogen, ammonia, nitrite, or reduced sulfur compounds. At first the interest in these organisms centered around thermodynamic aspects, principally a comparison of the relative efficiencies of chemo- and photosynthetic CO_2 assimilation. Studies of this sort have not materially contributed to our understanding of the mechanism of CO_2 utilization (cf. 71). In

one respect they are, nevertheless, of great significance for the present purpose since they have shown that chemical energy derived from the oxidation of many molecules of the inorganic substrates can actually be accumulated and used for the assimilation of a very much smaller number of CO_2 molecules. Hereby is revealed a definite similarity with the photosynthetic process which also requires the pooling of small amounts of energy, each by itself inadequate to permit the reduction of a CO_2 molecule to the stage of carbohydrate.

More important as a building block in the construction of a "comparative biochemistry of photosynthesis" is, however, the very fact that these chemo-autotrophic bacteria do accomplish a transformation of CO_2 into organic matter—and do so without the benefit of radiant energy. The demonstrable existence in microorganisms of mechanisms for CO_2 assimilation that can function through a series of exclusively dark reactions consequently makes it possible to view with greater confidence the previously developed conclusion that in photosynthesis, too, the reduction of CO_2 proper must be brought about by a sequence of non-photochemical events.

There are, moreover, some compelling reasons for accepting this analogy between photo- and chemosynthetic processes as a sound basis for the adopted viewpoint. In algae, as well as in purple bacteria, the occurrence of both modes of CO_2 assimilation has been established. Gaffron, in 1939, succeeded in so modifying the metabolic activities of a culture of *Scenedesmus*, by anaerobic incubation in the dark, that, upon illumination, the organisms no longer produced oxygen but carried out a photosynthetic reduction of CO_2 with the aid of molecular hydrogen. Their photosynthetic metabolism had thus become essentially similar to that of purple bacteria. And algae so treated can also assimilate CO_2 in complete darkness while oxidizing molecular hydrogen with oxygen as the final acceptor (35, 36, 72).

Similarly, those purple bacteria that can photosynthesize with, e.g., hydrogen or reduced sulfur compounds as hydrogen donors, and are able to grow in the presence of air, may also be grown in darkness. It is then possible to observe multiplication of the bacteria due to the use of CO_2 as the one ultimate carbon source for the synthesis of cell materials, the necessary energy being supplied by the oxidation of the inorganic substrates with atmospheric oxygen (34, 73).

Hence the composite of the above-mentioned facts conclusively demonstrates not only that various chemo-autotrophic microorganisms are endowed with a mechanism for reducing CO_2 by means of exclusively dark reactions, but furthermore that such mechanisms are encountered in normally photosynthetic organisms. To be

sure, it does not necessarily follow that this same mechanism must therefore also be operative in their photosynthetic CO_2 assimilation. However, to postulate the existence of other mechanisms—for which there is as yet no evidence—appears, at the present moment, not only superfluous and contrary to the principle of parsimony; but also unwarranted in view of the various arguments favoring the conclusion that CO_2 assimilation during photosynthesis consists of a series of non-photochemical reactions.

THE ASSIMILATION OF CO_2 AS A GENERAL BIOCHEMICAL PHENOMENON

By adopting the comparative-biochemical approach to a study of the photosynthetic mechanism it would, then, become theoretically possible to gain a deeper understanding of the intricacies involved in the assimilation of CO_2 through a more detailed investigation of this process as exhibited by chemo-autotrophic organisms. Unfortunately, our present knowledge of this phase is still very rudimentary. The most important contributions have come from the imaginative experiments of Vogler *et al.* (74, 75) with the colorless sulfur bacterium, *Thiobacillus thiooxidans*.

Suspensions of this organism, supplied with sulfur in the complete absence of CO_2 , will oxidize the substrate to sulfuric acid. If, at the termination of this reaction, CO_2 is admitted, the latter is assimilated. Thus it was shown that the energy-yielding process, *viz.*, the oxidation of sulfur, can be separated in time from the assimilation of CO_2 . It was even possible to demonstrate the occurrence of the assimilatory reaction in the complete absence of oxygen following a preliminary period of sulfur oxidation. It is, therefore, evident that part of the energy released during the oxidation of the inorganic substrate must be stored in the cells in such a manner that it can be used at a later stage for the assimilation of CO_2 . During the period of sulfur oxidation inorganic phosphate appears to be converted into an organic phosphate ester, and an opposite change seems to accompany the CO_2 assimilation.

One may thus conclude that in the metabolism of *Thiobacillus thiooxidans* a cyclic change of phosphate compounds links the energy-yielding oxidation reaction with the energy-requiring assimilation process. And it is highly significant that the phosphate ester has been characterized as a specific adenosine triphosphate, *i.e.*, a member of a group of substances known for some years to be used by various types of organisms as integral components of systems that serve the purpose of energy transfer (76–81).

Despite this important discovery one cannot truly claim that any phase of the exact mechanism of CO_2 assimilation has thereby become much clarified. For a satisfactory comprehension on a chemical level it should be possible to describe this process in terms

of a detailed series of intelligible step-reactions: to designate the successive changes which a CO_2 molecule undergoes during its conversion into cell materials. But the nature of such stages cannot be inferred merely from the knowledge that a phosphate cycle is operative as an energetic link in a chemo-autotrophic organism. Apart from the suggestion that in photosynthesis, as in many other biochemical processes, specific phosphate esters are likely to play a role, these studies do not furnish much material for comparative-biochemical speculations.

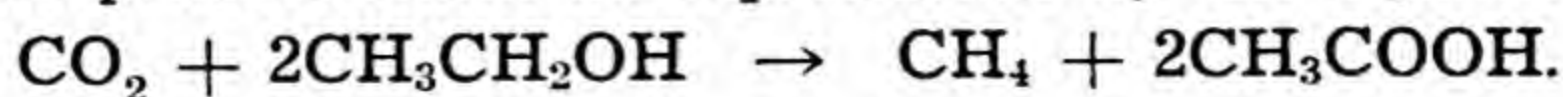
A more promising outlook is, however, afforded by the recognition that CO_2 reduction is not limited to the photo- and chemo-autotrophic organisms. Slightly over a decade ago four independent cases of CO_2 utilization by heterotrophic bacteria were, almost simultaneously, recorded. Since that time many additional instances have been discovered, and it is now no longer considered revolutionary to express the opinion that in the normal metabolism of any organism, whether microorganism, plant, or animal, some mechanism for CO_2 utilization is operative.

The four earliest examples of reactions in which heterotrophic bacteria carry out conversions of CO_2 can be segregated into two categories: (a) those in which CO_2 is reduced, but where the reduction product is still a one-carbon compound; (b) those in which CO_2 is built into an organic molecule, yielding substances with a longer carbon chain.

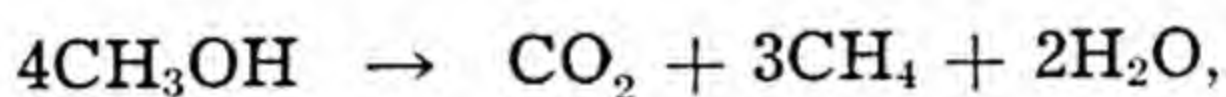
Reduction of CO_2

In 1936 Woods (82) observed that a cell-suspension of *Escherichia coli* is capable of producing formic acid from hydrogen and CO_2 . This is a reversal of the long-known reaction responsible for the formation of the two gases by decomposition of formic acid. Woods could show that this process is, in fact, reversible; in a closed system an equilibrium state is attained with the components present in nearly equal concentrations.

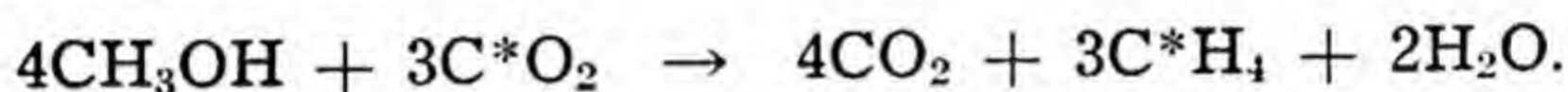
That same year Barker (15) demonstrated that the evolution of methane in the methane fermentation must be viewed as the result of the complete reduction of CO_2 . Such an interpretation was compulsory in the case of a methane fermentation in a medium containing ethanol and bicarbonate. During the development of the bacteria an almost quantitative conversion of ethanol into acetic acid occurred, accompanied by the disappearance of CO_2 . The extent of the latter corresponded with the amount of methane formed; the process could be represented by the equation:



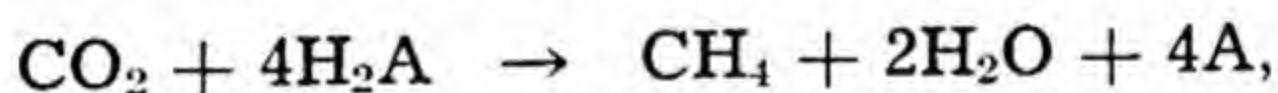
The incomplete oxidation of the organic substrate in this particular instance permitted an unambiguous interpretation of its fate, and hence also of the source of the methane. In most methane fermentations the only recognizable end products resulting from the decomposition of organic compounds are, however, limited to CO_2 and methane. One might interpret these processes, on the basis of comparative biochemistry, as complete oxidations of the organic substrate, coupled with a concomitant reduction of part of the CO_2 to methane. When carbon isotopes became available for biochemical studies, this extrapolation could be experimentally tested. By using isotopic CO_2 Barker *et al.* (16) verified the correctness of the inference, and showed that the methane fermentation of, for example, methanol, previously expressed by the equation:



should be represented by:



Similar results with acetate as a substrate support the contention that all methane fermentations can be generally formulated as:



thus providing evidence for the occurrence of a complete CO_2 reduction in darkness.

Condensation Reactions in Which CO_2 is Used to Build up Carbon Chains

Whereas the two above-mentioned examples of CO_2 utilization by heterotrophic organisms appear to be concerned with a straight reduction process of the one-carbon compound, the reactions to be discussed in this section are more nearly comparable with the CO_2 assimilation of autotrophs, since they provide direct evidence for the new-formation of carbon-to-carbon linkages, a prerequisite for the activities of organisms producing cell materials from CO_2 .

It was again in 1936 that Wieringa (83, 84) isolated an anaerobic bacterium capable of producing, in a typically catabolic process, acetic acid from CO_2 and H_2 . More detailed investigations concerning the mechanism of this mode of acetic acid formation have not yet been reported, but it has since been established that it is not restricted to Wieringa's *Clostridium aceticum*. Barker and Kamen (85) have made it very probable that part of the acetic acid produced in a glucose fermentation by *C. thermoaceticum* originates

from the linkage of two CO_2 molecules, since a fermentation conducted in the presence of C^{14}O_2 yields a considerable amount of acetic acid with the isotopic carbon in both the methyl and carboxyl positions. Similar results have been obtained in studies on the fermentations of *C. acidi-urici* and *Butyribacterium rettgeri* (86, 87). Present evidence thus supports the claim that the formation of a two-carbon molecule, acetic acid, from two molecules of CO_2 occurs in the metabolism of various types of heterotrophic organisms.

Furthermore, in some other organisms acetic acid must be formed by a sequence of reactions in which only one carbon atom of the two-carbon compound is derived from CO_2 since acetic acid isolated from fermentations in media with isotopic CO_2 contains the isotope exclusively in the carboxyl group. This has been reported for *Aerobacter indologenes* and *C. welchii* (88). The mechanism of this process appears closely related to the following example of CO_2 assimilation, also discovered in 1936.

At that time Wood and Werkman (89) announced that during a propionic acid fermentation of glycerol in the presence of carbonate appreciable amounts of CO_2 disappear from the system. Later (90, 91) it was found that this phenomenon is correlated with the appearance of succinic acid among the fermentation products, and that a close relationship exists between the quantity of CO_2 utilized and of succinic acid formed. By means of tracer studies (92, 93) it could be shown that CO_2 enters into the production of succinic acid, where it appears exclusively in the carboxyl groups.

Soon these observations were extended to metabolic processes other than the propionic acid fermentation. It was thereby established that succinic acid produced in the fermentations of bacteria of the coli-aerogenes group, in the metabolism of molds, of protozoa, and even of vertebrate tissues, is generally formed in part from CO_2 , and that the carbon of this substance is always located in the carboxyl group (cf. the general reviews by Werkman and Wood, 94, Krebs, 95, and Wood, 96).

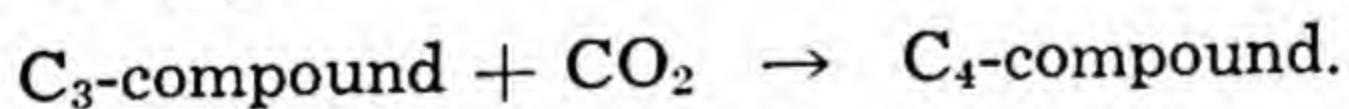
Far from being a process restricted to the metabolic activities of photo- and chemo-autotrophic organisms, the assimilation of CO_2 and its incorporation into organic molecules thus became recognized as a widespread phenomenon. Nor are acetic and succinic acids the only compounds in which assimilated CO_2 has been detected; propionic, lactic, pyruvic, acetoacetic, fumaric, α -ketoglutaric, oxalosuccinic, and citric acids are further specific examples of substances elaborated in this manner (94-96).

It will be evident that the regular occurrence in non-photosynthetic, and even heterotrophic, organisms of mechanisms permitting

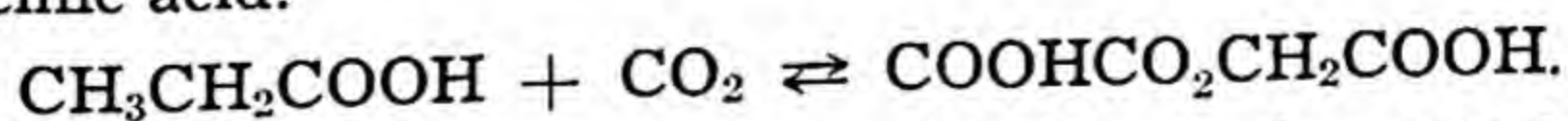
an assimilation of CO_2 by means of dark reactions considerably strengthens the case for the earlier deduction that the conversion of CO_2 in photosynthesis should be looked upon as a non-photochemical process. But the full significance of the facts enumerated in this last section does not emerge until it is appreciated that they may afford a clearer perception of the details of the mechanism whereby the conversion of CO_2 into cell materials is accomplished. In photo- and chemo-autotrophic organisms the observable result of the assimilation reaction is the formation of "cell materials," mostly of an unknown nature, and also of a vast complexity, comprising all the different carbohydrates, proteins, lipids, enzyme systems, etc., which together constitute a living cell. This very complexity makes a detailed analysis virtually impossible, unless one might, through the application of comparative-biochemical principles, once again surmise common denominators. And if, in particular organisms or under special conditions, such "fundamental" reactions can be found to proceed in a simpler and consequently more readily analyzable manner, their study will materially aid in developing reasonable working hypotheses for unraveling the complicated, over-all event. It is precisely the CO_2 assimilation by heterotrophs resulting in the formation of carboxylic acids which may be so considered, since present knowledge of these reactions is sufficiently advanced to grant a fairly clear comprehension of their mechanisms.

THE MECHANISM OF CO_2 ASSIMILATION

The early investigations of Wood and Werkman had established with reasonable certainty that the assimilation of CO_2 in the propionic acid fermentation should be ascribed to the occurrence of a reaction of the type:

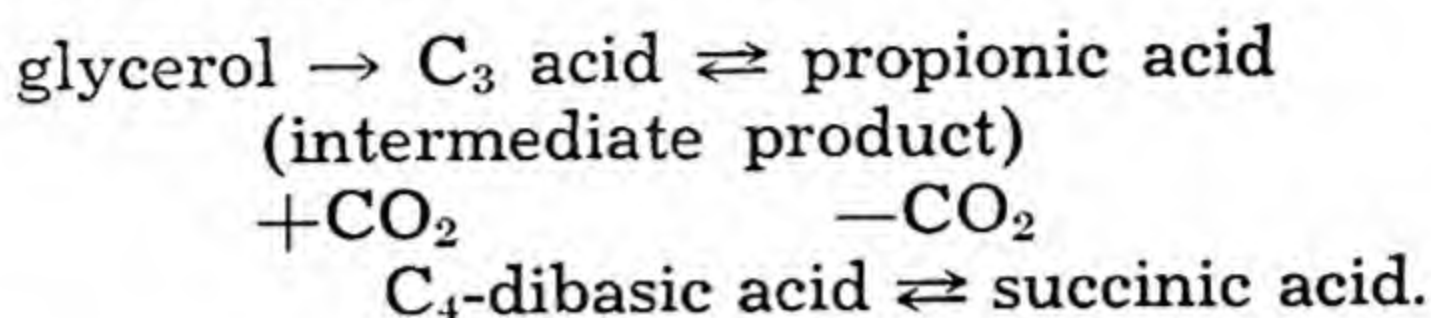


Since the substance with four carbon atoms was identified as succinic acid, it appeared possible to deduce the nature of the C_3 -compound. And when Carson and Ruben (92) found that the propionic acid formed during the fermentation of glycerol in the presence of CO_2 labeled with radioactive carbon was also radioactive, it looked as if the results could be quite simply explained by postulating a reversible reaction between propionic acid, CO_2 , and succinic acid:

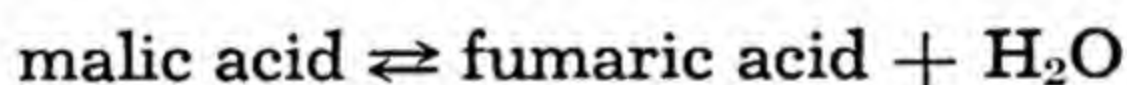


A logical consequence of this mechanism is that propionic acid bacteria, suspended in either propionate or succinate in the presence of radioactive CO_2 , should produce radioactive propionic and succinic acids. Special experiments were conducted to test this point;

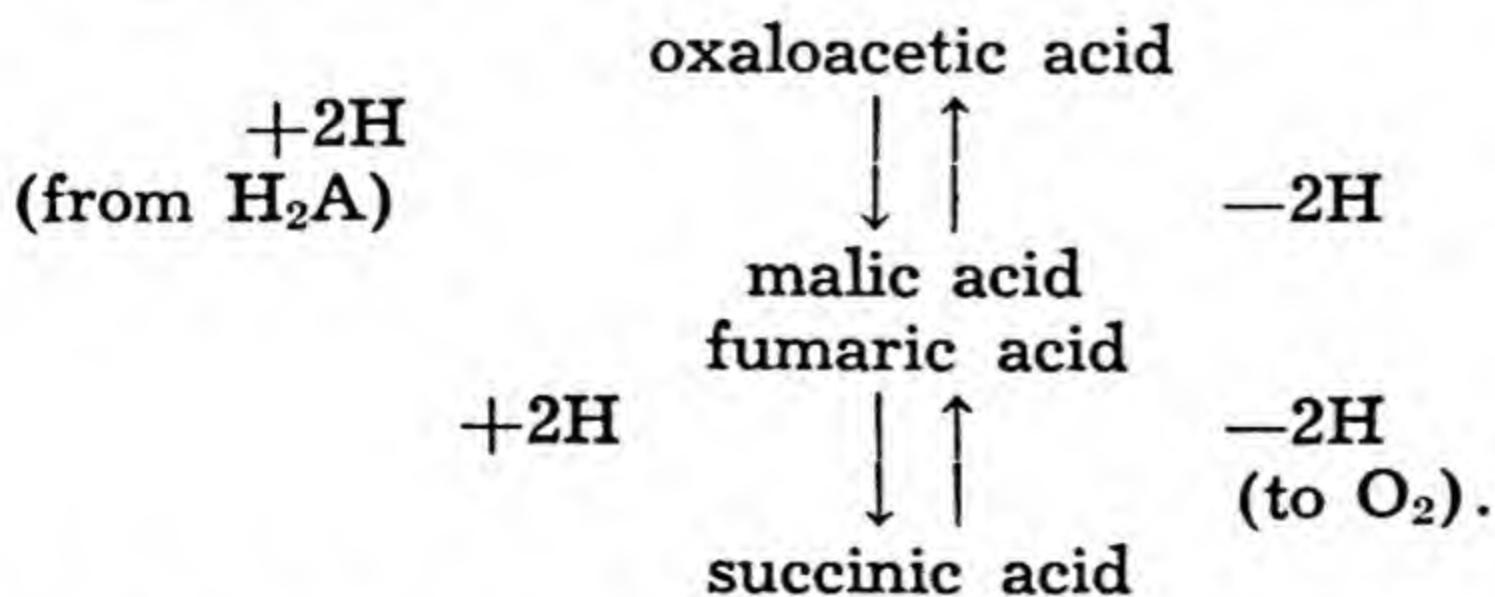
the results failed, however, to confirm the expectation. It was therefore concluded that the results were consistent with a more elaborate mechanism involving additional compounds:



At that time succinic acid and related 4-carbon dicarboxylic acids had come to occupy an important position in concepts of the mechanism of oxidative metabolism. They were considered as an integral part of a system that functions in various organisms in the gradual transfer of hydrogen from substrate to oxygen. Particularly through the studies of Szent Györgyi and collaborators (97) the idea had found widespread acceptance that the group of dicarboxylic acids, comprising oxaloacetic, malic, fumaric, and succinic acids, served as a "shuttle mechanism." Oxaloacetic acid, accepting hydrogen from some hydrogen donor, yields malic acid. The latter, under the influence of a specific enzyme, is partly transformed into fumaric acid; in fact, the reaction:



represents an enzymatically controlled equilibrium. The two components of the mixture can further undergo an oxidation-reduction reaction in which malic acid, as hydrogen donor, is reconverted to oxaloacetic acid, with fumaric acid, the hydrogen acceptor, becoming reduced to succinic acid. Dehydrogenation of succinic acid with the reformation of fumaric acid is accomplished through the mediation of the cytochromes, the hydrogen thereby being transferred to oxygen. The net result is, then, the transfer of hydrogen from the initial donor, H_2A , to oxygen, with the 4-carbon acids functioning as intermediate acceptors and donors:

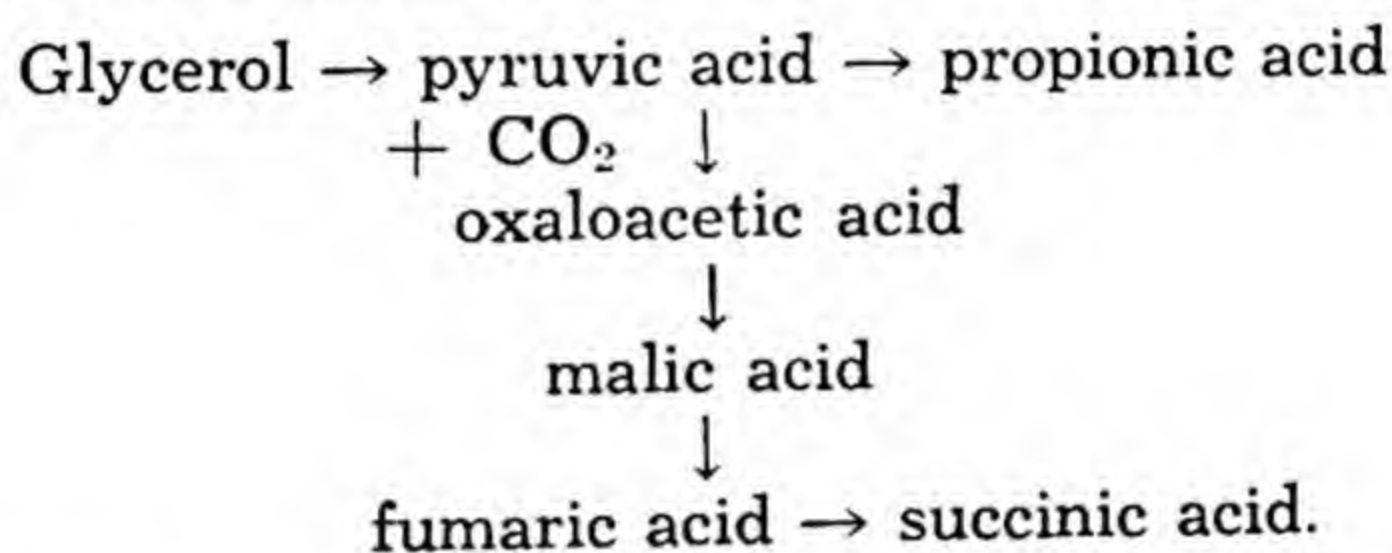


The most important experimental support for the occurrence of this "Szent Györgyi cycle" was the demonstration that cells which had become metabolically inactivated due to extensive

washing could be restored to a state of active metabolism by the addition of traces of any one of the four dicarboxylic acids.

Hence the studies on the Szent Györgyi cycle had revealed a mode of succinic acid formation of quite general occurrence, *viz.*, by reduction of oxaloacetic and fumaric acids. Could this knowledge be applied to the problem of succinic acid formation by propionic acid bacteria? The experiments of Krebs and Eggleston (98) made this very probable. After showing that each of the components involved in the cycle behaves in the predicted manner when acted upon by propionic acid bacteria they concluded:

"If pyruvic acid is substituted for C₃ acid and fumaric acid for C₄ dibasic acid the scheme of Carson and Ruben becomes almost identical with the following scheme comprising only reactions which have been shown to occur:



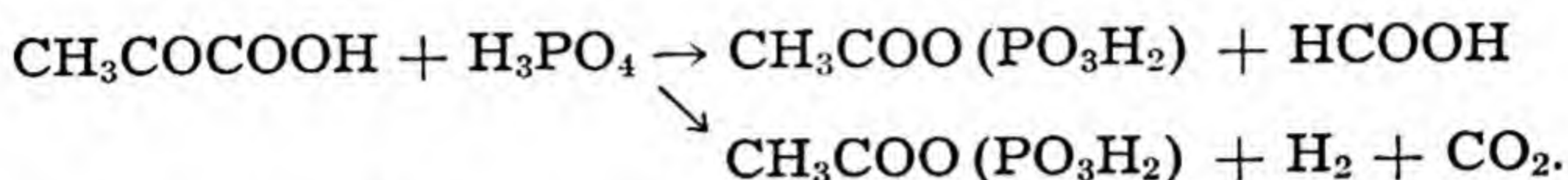
This scheme accounts for all the facts so far observed" (98, p. 685).

It will be seen that the crucial reaction of this scheme for CO₂ fixation is the formation of oxaloacetic acid from pyruvic acid and CO₂. The experiments of Krebs and Eggleston did not, it is true, supply direct evidence for its occurrence. Such evidence was, however, procured by later investigations with enzyme preparations from bacteria (99, 100) and from animal tissues (101).

This second example of CO₂ utilization by heterotrophic organisms thus appears, like the enzymatic synthesis of formic acid from CO₂ and hydrogen, to find its explanation in the reversibility of a reaction that previously had been observed to yield CO₂. The consequences of this discovery were far-reaching. It now became tempting to pose the question: Might not other biochemical processes, hitherto known to result in CO₂ production, turn out to be reversible?

Most important among the reactions generally held responsible for CO₂ formation by various heterotrophic organisms is the decomposition of pyruvic acid. The first attempt to reveal the possible reversibility of a decarboxylation of this substance by yeast preparations (102) yielded positive results, although the amounts of CO₂ converted into pyruvic acid are small indeed. In the meantime the masterly experiments of Lipmann (76, 103)

had shown that by far the more common pathway for pyruvic acid decomposition is not the simple decarboxylation characteristic of yeast metabolism, but a variant known as "oxidative decarboxylation" which proceeds in the presence of phosphate and a hydrogen acceptor, and leads to the production of CO_2 , acetyl phosphate, and reduced acceptor. Special cases of this reaction are the decomposition of pyruvic acid by certain bacteria according to the equations



Strict reversibility has been demonstrated by Utter, Lipmann, and Werkman (104) for the former of these two processes, and made extremely probable for the latter (104, 105).

Furthermore, the decarboxylation of some other keto acids has also been found to be reversible, and two of these are of particular significance for an understanding of the general mechanism of CO_2 assimilation. They concern the fixation of CO_2 in ketoglutaric and oxalosuccinic acids (96, 106, 107).

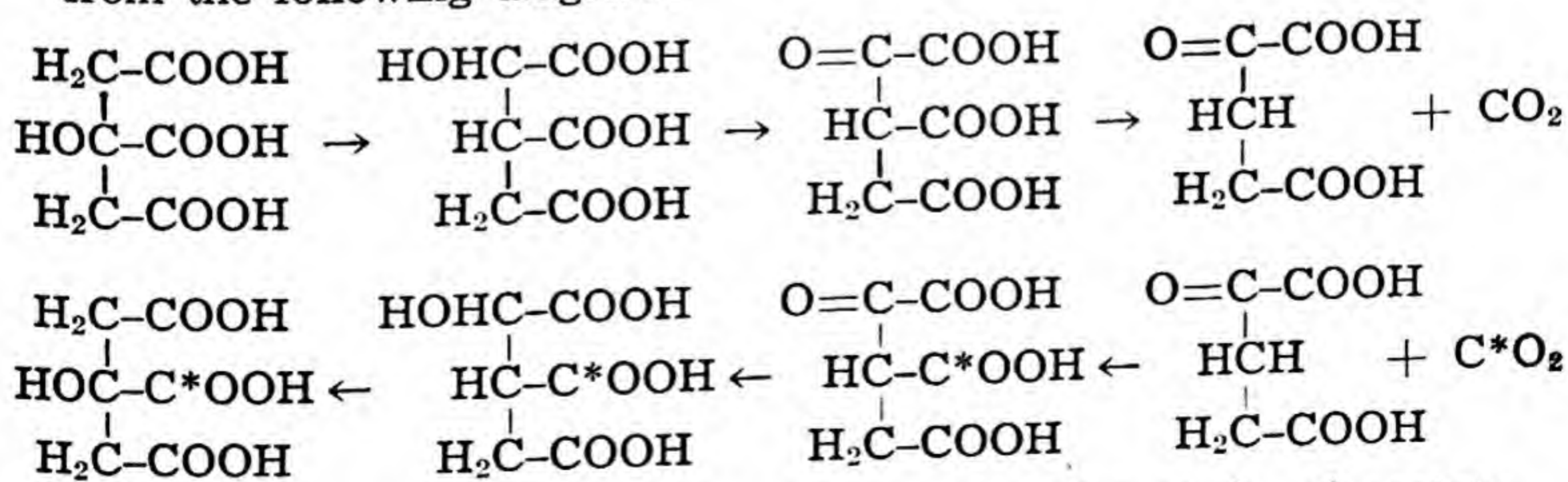
So far this discussion of the mechanism of CO_2 fixation by heterotrophs has served to bring out the principle that in a large number of cases the essential characteristic involved is a reversible carboxylation-decarboxylation reaction. The one outstanding example that does not seem to fit this pattern is the synthesis of acetic acid from CO_2 mentioned in the preceding section. Even this process can, however, also be interpreted on the basis of such a mechanism.

To Krebs (108) we owe the important discovery that the oxidation of acetic acid does not proceed by a simple dehydrogenation. In fact, the structure of the acetic acid molecule appears singularly unsuited for such attack. This difficulty was largely resolved when Krebs formulated the concept that, instead of being oxidized directly, acetic acid is first combined with oxaloacetic acid to form a 6-carbon tricarboxylic acid. This substance, under the influence of specific enzymes, in reality is represented by an equilibrium mixture of *cis*-aconitic, citric, and isocitric acids. The isocitric acid can be readily dehydrogenated to oxalosuccinic acid which, being a keto acid, is subject to decarboxylation with the formation of CO_2 and α -ketoglutaric acid. An oxidative decarboxylation of the ketoglutaric acid then yields CO_2 and succinic acid, the latter compound in turn being dehydrogenated with the regeneration of oxaloacetic acid. The net result of this series of steps is the production of 2 mols each of CO_2 and H_2O from one mol of acetic acid, which is equivalent to a complete oxidation.

This mode of oxidation of acetic acid implies that the two mols of CO_2 evolved are here too derived from decarboxylation processes. With the knowledge that both of these reactions are reversible, the possibilities for the formation of acetic acid through carboxylations, i.e., through a reversal of the "Krebs cycle," are obviously present. A reductive carboxylation of succinic to α -ketoglutaric acid, followed by a carboxylation of the latter and reduction of the ensuing oxalosuccinic acid would lead to the formation of isocitric acid. Through a decomposition of its conversion product, *cis*-aconitic acid, the assimilated CO_2 could be split off as acetic acid, accompanied by oxaloacetic acid. In view of the reducing conditions in the cultures of the anaerobes that are known to produce acetic acid from CO_2 , the rapid reduction of oxaloacetic acid back to succinic acid, as well as the occurrence of a reductive carboxylation and reduction of oxalosuccinic acid can certainly be expected.

Application of the concept that the two decarboxylations in the citric acid cycle are reversible also makes it possible to explain some additional observations which otherwise are difficult to understand. One of these pertains to the formation of citric acid by molds, the other to the decomposition of acetic acid by purple bacteria.

In 1941 Foster *et al.* (109) found that the citric acid produced from sugar by a culture of *Aspergillus niger* in the presence of CO_2 labeled with radioactive carbon contained the carbon isotope, and this exclusively in carboxyl groups. This result shows conclusively that CO_2 had entered into the synthesis of the tricarboxylic acid. But the mechanism of citric acid formation proposed by Krebs does not, at first sight, call for the participation of CO_2 . One might, therefore, feel inclined to postulate a different mechanism for citric acid production in this particular case. That such is not necessary appears from the fact that a reversible decarboxylation of oxalosuccinic acid accounts equally well for the recorded facts, as is evident from the following diagram:

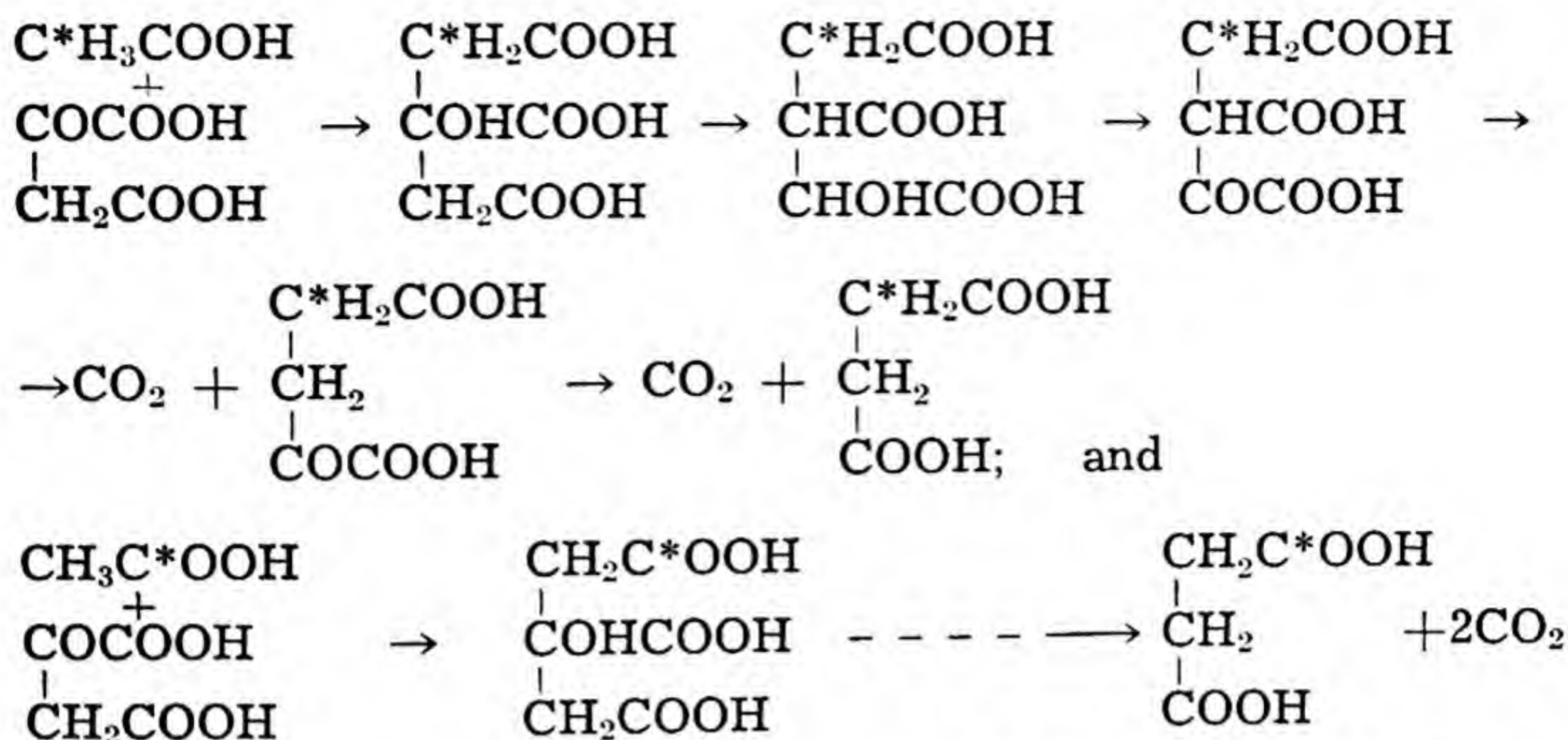


Of even greater interest are the results of a study of acetate oxidation by non-sulfur purple bacteria in the dark. In the first

place this oxidation was known to proceed only in the presence of CO_2 (34, p. 305-06); if the system is kept free of CO_2 by the inclusion of alkali the substrate is not attacked. This striking phenomenon is strongly reminiscent of the observations made by Hes (110, 111) who reported that methylene blue reduction by various microorganisms is markedly inhibited by exclusion of CO_2 from the medium. It has recently been shown (unpublished experiments) that the addition of traces of succinic, fumaric, malic, or oxaloacetic acid will permit the rapid oxidation of acetate by purple bacteria even in the absence of CO_2 . This provides the first experimental support for the thesis (112) that complete elimination of CO_2 would interfere with normal metabolism by exhausting the supply of dicarboxylic acids through a continued decarboxylation of oxaloacetic acid. It has thus been ascertained that acetate oxidation by the purple bacteria requires the presence of 4-carbon dicarboxylic acids. This, in turn, suggests that the decomposition of acetate is effected through the operation of the Krebs cycle.

It has also been shown previously (34) that during the oxidation of acetate a considerable proportion of the substrate is converted into cellular substances. In an attempt to determine experimentally which part of the acetic acid molecule is specifically involved in this synthetic process, further studies have now been carried out in collaboration with Dr. H. A. Barker, of the University of California (unpublished). For this purpose advantage was taken of the availability of acetic acid labeled with radioactive carbon (C^{14}), either in the methyl group, or in the carboxyl group, or in both positions. It will be obvious that it should be possible to infer the fate of the two halves of the acetic acid molecule from an estimation of the amounts of radioactive carbon liberated as CO_2 and stored as cell material respectively, and relating this information to the known composition of the initial substrate.

So far, the experimental results indicate that close to one-half of the total radioactive carbon of the decomposed acetic acid is recoverable in the form of CO_2 , and that an approximately equal amount is stored as cellular constituents, irrespective of the original position of the labeled carbon in the substrate molecule. This implies that the two carbon atoms in acetic acid are equivalent from the point of view of their convertibility into cell materials and CO_2 . Again, this would be readily understandable if acetic acid were oxidized via the Krebs cycle, because in that case the carbon atoms of acetic acid will gradually become incorporated into succinic acid which, after conversion to oxaloacetic acid, starts off a new cycle:



In complete agreement with the above deductions are the results of investigations (with Dr. H. A. Barker, unpublished) in which radioactive succinic acid was isolated both from cultures of *Rhodospirillum rubrum* oxidizing succinic acid in the presence of radioactive CO_2 , and from cultures oxidizing isotopic acetic acid in the presence of non-labeled succinic acid. In the first type of experiment the radioactivity recovered in succinic acid was, though unmistakable, relatively small; in the second type a very considerable proportion of the isotopic carbon from acetic acid was found in the succinic acid which could be recovered at the end of the run.

In spite of these undeniably suggestive results, a word of caution is here in order. This is due to the fact that some observations seem, as yet, to contradict the occurrence of the tricarboxylic acid cycle as operative in the oxidation of acetic acid by *R. rubrum*. In the first place, this bacterium has been found incapable of growing in media where citric acid represents the chief carbon source (73). Furthermore, α -ketoglutaric, citric, and isocitric acids do not exert the expected catalytic effect on the oxidation of acetic acid by this organism under conditions in which the 4-carbon dicarboxylic acids are active in this manner. Nevertheless, it seems to me premature to postulate a participation of the succinic acid group of compounds different from that embodied in the Krebs cycle; it is quite possible that future studies may show that some slight modification of the experimental conditions has a profound effect on the outcome of such experiments.

Summarizing the argument developed in this section, it can be stated that there exists a large body of evidence supporting the thesis that the primary mechanism of CO_2 fixation by heterotrophic organisms in darkness consists of a carboxylation reaction.

It is even probable that future investigations will show that all processes in which CO_2 can be evolved in metabolism are reversible, thus demonstrating many means for the assimilation of this compound. Hence the question may be asked: Do such reactions have any application to the process of photosynthesis?

Since the available information strongly points towards the participation of CO_2 in a dark reaction, it seems logical to answer this question in the affirmative. Besides, there is some experimental evidence that, also in photosynthesis, the initial reaction in which CO_2 is involved represents a carboxylation. Shortly after McAlistier (113) had demonstrated that green plants fix CO_2 in darkness immediately following a period of illumination, Ruben *et al.* (114) found, by the use of isotopic CO_2 , that the product of this fixation is probably a carboxylic acid.

Already in 1938 Thimann had developed this line of reasoning in a remarkable publication (115). He proposed that, because photosynthesis is in many respects the reverse of respiration, nothing would be "more natural than to suppose that in photosynthesis the absorption of CO_2 takes place in the reverse way (from that in which CO_2 is produced in oxidations and fermentations), by combination with an aldehyde, or, more probably, with an organic acid, to produce a new carboxyl group." Later developments in our understanding of oxidative metabolism, leading to the recognition of the Krebs cycle, have been embodied in the more specific hypothesis recently advanced by Ochoa (107), in which the tricarboxylic acid cycle plays a major role in the assimilation of CO_2 . Ochoa, too, concludes that "the processes of both photosynthesis and chemosynthesis may represent reversals of the respiratory process not only from the standpoint of energy but also from the standpoint of enzymic mechanisms." In this connection one might point to the general occurrence in plants of members of the Krebs cycle, such as malic, citric, and isocitric acids, often in very large amounts, as supporting these contentions. It is interesting to reflect on the fact that, more than a century ago, the presence of such compounds induced Liebig to formulate his concept of the mechanism of photosynthesis, postulating a gradual reduction of CO_2 to sugar through the stages oxalic, tartaric, malic, and succinic acids. "Alles ist neu, und doch immer das Alte!"

Experimental support for the postulated fixation of CO_2 in higher plants by dark reactions of the sort involved in the Krebs cycle is not lacking. Gollub and Vennesland (116) showed that parsley roots can produce malate from pyruvate by CO_2 addition; Vennesland, Ceithaml, and Gollub (117) extended these studies, demonstrating the formation of tricarboxylic acids from CO_2 and α -ketoglutaric acid by the same plant material.

It is true that these processes are exhibited by chlorophyll-free, and hence non-photosynthesizing parts of plants. Thus the objection might be raised that in the chlorophyllous tissues the mechanism of CO_2 assimilation is fundamentally different. It is therefore important that Benson and Calvin (118, 119) have carried out investigations with *Chlorella* cells under conditions which guarantee the operation of a true photosynthetic mechanism, and that the results provide strong evidence for the view that the same or very similar steps are here also involved in the assimilation of CO_2 . It would seem that the problem of the mechanism whereby CO_2 is converted into cell material during photosynthesis is rapidly approaching a solution.

No matter how attractive these ideas may appear from the point of view of comparative biochemistry, they have not remained undisputed. In a recent paper Allen, Gest, and Kamen (120) have listed a number of objections to the hypothesis that CO_2 fixation in photosynthesis proceeds by way of the now recognized major types of carboxylations exhibited by heterotrophic cells. In the first place they point out that the molecular weight of the "carboxylic acid" studied by Ruben *et al.* (121) is too great to fit into such a scheme. Secondly they claim that in *Chlorella pyrenoidosa* and *Scenedesmus* sp. (Gaffron's strain D3) none of the usual intermediates which have been demonstrated to be active in heterotrophic CO_2 -fixation are found to participate in dark CO_2 -fixation. And finally the authors state that "the sensitivity of the dark fixation to various inhibitors parallels the over-all inhibition of photosynthetic oxygen evolution and not respiratory oxygen uptake."

If these arguments were based on completely satisfactory experimental evidence, attempts to identify the primary fixation reaction with any of the known heterotrophic carboxylations would, of course, have to be abandoned. However, it seems to me that the studies of Allen *et al.*, do not compel such a conclusion.

In the first place, the algal suspensions were exposed to the isotopic CO_2 without an immediately preceding illumination—in the case of starved cells even after prolonged storage in darkness—and for an excessively long time. This procedure should be expected to result in a distribution of the assimilated carbon over a great variety of compounds, all arising in a multitude of dark reactions. It is thus *a priori* improbable that the primary assimilation product would be present in more than trace amounts at the end of the treatment. Furthermore, the mixture of acids used as carriers for the isolation of specific substances lacked various well-known components of the Krebs cycle, such as succinic, malic, oxaloacetic, isocitric, *cis*-aconitic, and oxalosuccinic acids. Had these acids been present among the radioactive products of CO_2 fixation, the

procedure followed would have failed to detect them. That under these circumstances small but measurable quantities of fumaric acid and of keto acids (pyruvic and α -ketoglutaric acids) were isolated indicates clearly that the above-mentioned cycle must have been in operation, though it obviously cannot be decided whether the dark reactions responsible for the results are in any way connected with the photosynthetic process. At any rate, the failure to identify the bulk of the radioactive material produced in the experiments does not constitute a valid reason for eliminating the Krebs cycle as a possible mechanism for the entrance of CO_2 in photosynthesis.

Nor are the published results of the cyanide experiments any more conclusive. In fact, the statement that the dark fixation in the presence of cyanide parallels photosynthesis rather than respiratory oxygen uptake is true only for *Scenedesmus*; in the case of *Chlorella* the relation is rather the reverse. Here cyanide, in sufficient concentration to inhibit photosynthesis almost completely, has but a moderate effect on CO_2 fixation. Moreover, the response of *Scenedesmus* to cyanide does not justify the conclusion that the dark fixation of CO_2 in photosynthesis must be distinct from that in heterotrophic organisms. That low concentrations of cyanide abolish the oxygen consumption by this alga does not imply that none except those dark reactions directly associated with the photosynthetic mechanism are left fairly intact. Ever since the investigations of Wieland it has been known that, while cyanide may prevent oxygen utilization, its presence does not eliminate all oxidative processes. In fact, most biological oxidations can proceed practically unimpaired in the presence of cyanide provided that an appropriate hydrogen acceptor other than oxygen be present, and fermentations as a rule are not much influenced by cyanide. Hence a cyanide-poisoned *Scenedesmus* suspension is likely to carry out a variety of reactions regularly concerned in its normal dark metabolism. And the identification of fumaric and keto acids among the products of CO_2 fixation provides strong evidence that at least some reactions, recognized as an integral part of the Krebs cycle, had occurred.

What the experiments of Allen *et al.*, have shown is simply that CO_2 fixation by the two species of algae in darkness is not much affected by cyanide, and consequently one may conclude that the fixation reaction probably does not involve a heavy metal catalysis. These results cannot be used, however, as a valid argument against the concept that it is through a reversible step in the Krebs cycle that CO_2 enters into the formation of organic matter during photosynthesis. It is not my intention to defend the idea that this

mechanism must necessarily be responsible for the initial assimilation of CO_2 in photosynthesis. Admittedly, it is conceivable that a different mechanism might here be involved. Nevertheless, I believe that it is at present the most reasonable working hypothesis, against which compulsory experimental evidence has not yet been presented.

THE PROBLEM OF PRIMARY SYNTHESIS AND THE FORMATION OF ORGANIC MATTER BY PHOTOSYNTHESIS

Up to this point the comparative biochemical analysis of photosynthesis has shown that this process may be regarded as a photochemical decomposition of water with a subsequent reduction of CO_2 . The available evidence goes far to support the postulate that CO_2 , prior to its reduction, is incorporated into an organic molecule and that the mechanism of this preliminary fixation is analogous to if not identical with one or more of the known reversible steps in the Krebs tricarboxylic acid cycle. This fixation as well as the reduction of the carboxylic acid have been shown to occur in organisms not possessing photosynthetic mechanisms and there are many arguments in favor of the thesis that in photosynthetic organisms they also proceed as dark reactions.

In principle, such a formulation of photosynthesis is extremely simple. But this very simplicity implies that only the most general features have been considered. One only needs to appreciate the immense variety of organic substances which green plants and photosynthetic bacteria manufacture from CO_2 in order to realize that a description of photosynthesis in the above terms still leaves a great many problems unanswered. It is the purpose of the following discussion to contend that these problems are not restricted to the photosynthetic mode of life, and to indicate in what directions advances have been made to aid in their solution.

At the outset it must be realized that the ability of green plants to synthesize their multitude of cellular constituents from a single carbon source is not a prerogative of photosynthetic metabolism. A great variety of microorganisms are known to thrive in complete darkness in media containing only one single organic substance. They must consequently derive the carbon for the manufacture of their cell material from this substrate alone. Furthermore, the same organism can often utilize diverse organic compounds in this manner. Particularly impressive are the results of den Dooren de Jong's investigations which have shown that *Pseudomonas putida*, for example, can develop with any one of 77 different organic compounds belonging to the groups of primary alcohols, saturated and unsaturated fatty acids, hydroxy and keto acids, di- and tribasic

acids, polyalcohols, sugars, amines, amides, amino acids, and aromatic compounds (122). And this is merely one instance out of many. It is not difficult to agree with Kluver's appraisal of such facts: "Here we find the biochemical miracle in its fullest sense, for we are bound to conclude that all the widely divergent chemical constituents of the cell have been built up from the only organic food constituent" (10, p. 13).

In all these cases the synthesis of cell material is indubitably the result of metabolic processes which occur without the benefit of a supply of radiant energy, *i.e.*, exclusively through a succession of dark reactions. The existence of so many organisms capable of effecting such transformations thus leads to the question whether photosynthetic organisms, also, might not manufacture the numerous organic substances which they contain by means of similar dark reactions, starting from one or a few key substances which are themselves the immediate and only results of photosynthesis. If this were so, a better understanding of the mechanism whereby the various plant products are formed might be gained equally well through studies on the formation of such compounds in non-photosynthetic cells.

It is a well-known fact that many algae which can be grown in pure culture in a strictly mineral medium—where they produce their cellular components by means of photosynthesis—can develop equally well in darkness if provided with a single organic carbon source, such as acetate or sugar. This holds true also for the photosynthetic bacteria (73). Still more to the point are examples drawn from the higher plants. Here, the successful culture of isolated roots in sugar-containing media has shown convincingly that these organs, when shut off from the supply of such organic matter as is normally furnished by the photosynthetic elements of the plant, can manufacture all or nearly all of their cell material from a single carbon compound. And, finally, reference should be made to the important experiments of Spoehr on the culture of albino maize (123) which investigations have proved that a large plant can result from the transformations of glucose or sucrose as the only carbon source by means of exclusively non-photochemical reactions. These examples demonstrate that the production of cell constituents can be accomplished by plants without benefit of light, from a single organic substance. They would also lead to the conclusion that even in higher plants photosynthesis proper may well be confined to the formation of only one organic compound which can be designated as *the* typical product of the photosynthetic reaction.

In the past much effort has been devoted to a search for such a product. The accumulated experience with green plants has

tended to support the belief that it is a carbohydrate. There are two lines of evidence for this:

1. The ratio between the amounts of CO_2 assimilated and of oxygen produced in green plant photosynthesis is, in most cases, close to unity. This can only mean that the other product of the photosynthetic reaction has an over-all composition which must be essentially equal to that of carbohydrate.

2. The actual formation of carbohydrate can be easily demonstrated. A convincing qualitative proof is furnished by the classical test in which starch production can be observed after using iodine on a leaf that has been illuminated for some time. More recently Smith (116) has published quantitative data on carbohydrate formation in sunflowers during photosynthesis. From these it can be concluded that starch is not the principal assimilation product in short-term experiments, but rather the disaccharide, sucrose. Nevertheless, the results leave no doubt that practically all of the assimilated CO_2 can be accounted for by the increase in total carbohydrates.

Preoccupation with this carbohydrate concept has been responsible for much discussion of the question whether the first product of photosynthesis is a simple hexose, a disaccharide, or a polysaccharide. No matter how intriguing this problem may appear, it seems to me that even the postulate of a primary formation of carbohydrate is probably not of general applicability. Many of the instances in which a close correlation between photosynthesis and carbohydrate formation has been established are concerned with relatively complicated organisms, exhibiting a distinct separation of function between different parts. This obviously requires a mechanism for the transport of materials from one part of the organism, where they are either present or manufactured in abundance, to other parts, where they are used for normal metabolic activity. In the higher plants this implies the need of transporting synthesized organic compounds from leaves to stems, roots, flowers, and fruits. It is readily understandable that carbohydrates represent probably the most satisfactory form for the transport of large quantities of organic matter. Their general solubility characteristics, the relatively small size and ease of penetration of the mono- and disaccharides, and the ready convertibility of the latter into insoluble polysaccharides, coupled with their electrochemically neutral behavior, all make them appear singularly well-suited for the purpose. This is supported by the fact that in animals, too, these substances play the same role. However, this does not imply that carbohydrates are the first, nor necessarily the only immediate products of photosynthesis; it is at least conceivable that they might arise from the

conversion of some other primary assimilation product, just as glycogen formation in muscle tissue may result from the oxidation of lactic acid.

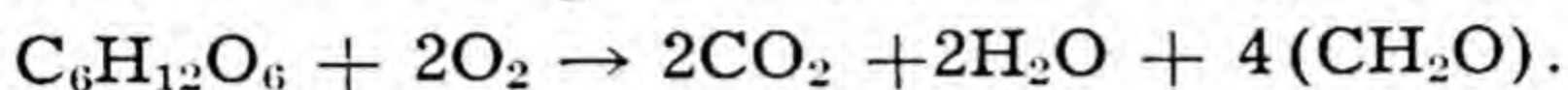
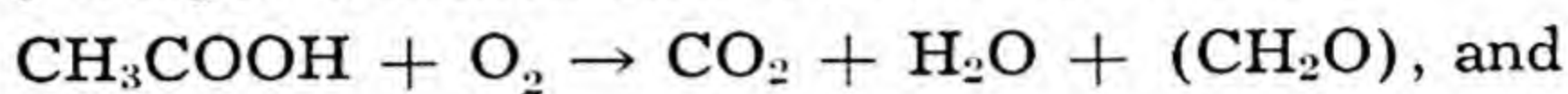
In contrast to the rather clear-cut cases involving carbohydrate as a product of photosynthesis there are others in which the evidence is much more indicative of the formation of different substances. Especially among the photosynthetic reactions carried out by the purple bacteria this is the rule rather than the exception. Here the ratio of assimilated CO_2 to oxidized hydrogen donor is often appreciably smaller than unity. With the most accurate methods available it has generally been found that the assimilation product is more reduced than carbohydrate. Determination of the ratio in which hydrogen and CO_2 are used by photosynthesizing, resting cell suspensions of purple bacteria—theoretically 2.0 for the formation of carbohydrate—have yielded values of 2.4 to 2.6 (125). Similar data are available for bacterial photosyntheses with other substrates (34).

It is, of course, a simple matter to reconcile such observations with the hypothesis of a primary carbohydrate formation by assuming that secondary reactions are responsible for a rapid conversion of carbohydrates into more reduced compounds. However, it is not necessary to resort to this explanation, and it seems to me preferable to adopt a broader view. This view is largely based upon a comparative-biochemical consideration of the situation in photosynthesis and of present-day knowledge concerning that phase of microbial metabolism which has been designated by the terms oxidative and fermentative assimilation.

About ten years ago Barker (126) observed that resting cell suspensions of the colorless alga, *Prototheca zopfii*, responded to the addition of various oxidizable substrates by rapidly consuming oxygen, but that the quantity of oxygen so utilized remained very much below that necessary for the complete oxidation of the amount of substrate added. The substrate had, nevertheless, disappeared, and no evidence could be found for the formation of oxidation products other than CO_2 . These results were interpreted to mean that the fraction of the substrate not accounted for in the form of CO_2 had been converted into cellular substance by some sort of primary assimilation process. The quantitative relations between substrate supplied, oxygen consumed, and CO_2 formed made it possible to compute the over-all composition of the assimilation product; it appeared to be that of a carbohydrate. It seemed reasonable to suppose that the assimilation product was a reserve food material stored in the cells.

The results further showed that the extent of this primary

assimilation was considerable; only from one-half to one-third of the substrate-carbon could be recovered as CO_2 , the amount depending upon the nature of the substrate. The oxygen consumption was in excellent agreement with so incomplete an oxidation. The most remarkable feature was that the conversion of substrate into CO_2 and cell material or reserve product could be expressed by amazingly simple stoichiometric equations, such as:



Soon afterwards it was established that oxidative assimilation is of widespread occurrence. In fact, it has been encountered in every case where the experimental setup was appropriate for its detection. In addition, it was found that similar assimilations may occur under anaerobic conditions; for these the term "fermentative assimilation" has been used (cf. Clifton, 127).

The stoichiometric relations which Barker had determined for *Prototheca* appeared to fit many of the data obtained with various other microorganisms. In those cases where the quantitative results were obviously different some other equally simple equation could generally be proposed as an adequate description of the process. Naturally, such results raised the hope that further studies of this phenomenon would yield important information concerning the mechanism of assimilatory processes.

It was, of course, obvious that some biosynthesis had to be accomplished by an organism actually growing at the expense of the oxidation of a single organic substrate. But it is extremely difficult to analyze the synthetic activities under these conditions. In the first place, the relative extent of synthesis as compared with the amount of substrate decomposed is, at least in bacterial cultures, generally quite small. In this respect the molds appear to behave differently; as Waksman has frequently stressed (*e.g.*, 128, p. 513 ff., 702 ff.), it is not unusual to observe a 50 per cent conversion of an organic nutrient into mold-cell material. It is gradually becoming clear, however, that bacteria are not necessarily less efficient. The reason for the earlier contrary impression must be sought in the often overlooked fact that in bacterial cultures the oxygen provision is apt to become limiting long before the concentration of the oxidizable substrate does; this difficulty is not encountered in mold cultures where growth usually occurs in the form of a mat on the surface of the culture medium with adequate access to the air. By providing conditions of better aeration or by using low substrate concentrations, experimental conditions can, therefore, be provided where the difficulty inherent in an apparently low efficiency

is eliminated. It is not possible, however, to dismiss equally simply the second and major obstacle involved in an analysis of biosyntheses with growing cultures. Here the substrate molecules are converted into an enormous diversity of substances, and a better understanding of the mechanism of such transformations can hardly be expected until one can study the synthesis of a single compound at a time. Hence biochemists have generally contented themselves with the statement that the synthetic, or anabolic, reactions are energetically coupled with those causing the breakdown of the substrate (catabolic processes). The energy released by the latter would in part be used for the synthesis of cell material.

A serious attempt at developing a concept of biosyntheses that extends beyond this general formulation, and permits a more satisfactory comprehension in a chemical sense, was made by Kluyver (10-12). He envisaged the possibility of a simple enzymatic synthesis of cell constituents, not directly from the substrate itself but from certain intermediate products arising during its breakdown. Catabolism could then more accurately be represented as a means of providing the cell with appropriate building blocks, rather than as a mere energy-furnishing process. The nature and amount of the building blocks—these being intermediate catabolic products—would then largely be determined by the structure of the substrate molecule, and the latter might be far more important for the concomitant synthetic reactions than the amount of energy that can be derived from catabolism. An experimental verification of this consequence was, however, delayed until the synthesis of a single substance could be studied.

The discovery of the phenomenon of oxidative assimilation indicated the possible usefulness of resting cell suspensions, supplied with a single substrate, for the purpose of investigating the biosynthesis of some specific compound. The absence of nitrogenous food sources here precludes the synthesis of proteinaceous substances and is likely to limit the synthetic processes to the production of reserve foodstuffs; the short duration of the experiments suggested that these syntheses might even be confined to the formation of a single assimilation product; the simple stoichiometric relations could reasonably be considered as evidence that this was actually the case.

Thus the stage was set, and significant experiments were soon reported. Clifton and Logan (129), using suspensions of *Escherichia coli*, showed that the quantity of carbon assimilated from acetate, succinate, and fumarate amounted in each case to one out of every four carbon atoms decomposed. Lactate and pyruvate also yielded comparable results, although here one out of every three carbon

atoms appeared to be converted into the primary assimilation product. In experiments with *Pseudomonas saccharophila* Doudoroff found that two out of every three carbon atoms were assimilated from glucose, lactate, and pyruvate.

These initial results seemed indeed promising. It was clearly impossible to account for them by mere considerations of energetic relations; in that event the amount of synthesized reserve material should have been greatest when acetate or sugar was oxidized because: (a) more energy per carbon atom is liberated in the oxidation of these substances than from the other members of the group, and (b) less energy is required for their conversion into a common assimilation product.

Furthermore, it might be inferred that a carboxyl group in the substrate is unsuitable for synthetic purposes and is eliminated. Such a conclusion is supported by the fact that during the oxidation of acetate by the colorless alga, *Prototheca*, by yeast, and by a number of bacteria, one-half of the substrate is assimilated. In these cases, as also in the above-mentioned ones concerning lactate and pyruvate oxidation, the rest of the organic acid molecule might then have been converted entirely into the storage material. Other organisms, less "efficient," seemed to carry out a degradation process by different steps, so that only in a later stage the proper building block for synthesis emerged, resulting in a greater loss in the form of CO_2 .

Nevertheless, this simple and attractive picture must today be viewed with grave misgivings. In the first place, the quantitative relations between substrate, oxygen, and CO_2 often made it quite obvious that the assimilated material did not correspond to carbohydrate, but was appreciably more reduced. Even more serious is the fact that much the same extent of assimilation as that observed with resting cell suspensions was exhibited by growing cultures of both *Escherichia coli* and *Pseudomonas saccharophila* (129, 130). Certainly more than just carbohydrate was being synthesized in such cultures. And if, as might be supposed, such syntheses represented secondary conversions of a primary assimilation product, one would expect that each of these transformations would involve losses in carbon of the same order of magnitude as those attending the primary assimilation process, a consequence clearly contradicted by the experimental results. And, finally, unpublished experiments on the decomposition of acetate by non-sulfur purple bacteria in darkness have shown that far more than half of the substrate-carbon can be assimilated, thus demonstrating that carboxyl groups can be converted into cell material.

Does this mean that the approach developed by Kluyver must be

given up? Of course not. The contention of the above paragraph is not that this principle is unsound, but rather that the experiments on oxidative assimilation have: (a) failed to contribute results that can be used for determining the nature of the building blocks for synthetic reactions, and (b) indicated that organic compounds other than carbohydrate may arise as the first product of assimilation. Carbohydrate no longer seems necessary as a starting point for such syntheses; it now appears that the composition of the assimilation product may be much more varied than was at first assumed. It is highly probable that this conclusion is equally applicable to the problem of the production of organic substances by photosynthesis.

In this connection the recent work of Myers (131) on oxidative assimilation by *Chlorella pyrenoidosa* is of interest. The experiments were carried out with a view to examining the relationship between oxidative assimilation and photosynthesis. As in so many other instances the oxidation of acetate could be represented by the equation which Barker first established for *Prototheca*; the oxidation of glucose yielded less CO_2 , and apparently more assimilation product than had hitherto been observed in such studies with other microorganisms. It was further found that illumination of the algal suspensions did not affect the course of glucose or acetate assimilation, and from these and similar experiments Myers concluded that glucose cannot be an intermediate product of photosynthesis in *Chlorella*.

It must be granted that this conclusion does not preclude the occurrence of a mechanism in which some carbohydrate other than glucose functions as a primary assimilation product. Nevertheless, as will be discussed below, present trends in biochemical thought are more conducive to a different outlook. This is expressed by Myers in the statement: "With increasing knowledge of the biochemistry of the process the term 'product of photosynthesis' may in time become difficult of definition" (131, p. 225).

Considerable advances have been made in our understanding of the mechanism of biosyntheses during the past decade. These have indicated how a much more flexible concept can be developed in which the process of the formation of carbohydrate represents only one special case of a number of possible synthetic functions.

By far the most important contribution to the knowledge of biochemical syntheses has been the demonstration that practically all steps of the catabolic sequence are reversible. It seems justifiable to accept as a working hypothesis that every step can be so designated. Past failures to establish the reversibility of some partial reaction now known to be reversible have mostly been due to an inaccurate conception of the steps involved. In some cases it has

been ascertained that the intermediate products of catabolism are not, as was earlier supposed, the simple molecules that could be detected by appropriate techniques, but rather more unstable derivatives with greater chemical potentialities. Hence the genuine intermediate products were often converted into more stable substances by secondary reactions during the operations leading to their isolation. As a result the postulated mechanism for a catabolic reaction was frequently too simple, and individual steps, of crucial importance for reconstituting the reverse process, were omitted. In consequence the synthetic reaction could never be detected. An analogous situation is presented by the studies of certain plant glucosides. These, too, have often been isolated in the form of partial degradation products with the result that the pharmacological effects of the pure chemicals did not correspond to those of the crude plant extracts. Not until Stoll and his collaborators (132) developed new and much more delicate methods for the isolation of such plant products were the genuine glucosides obtained. And with these compounds the earlier discrepancies were no longer encountered. The following cases provide some striking examples.

After the Coris had discovered that the decomposition of glycogen in muscle tissue starts with a depolymerization which is not a hydrolysis but a phosphorolysis, the first genuine breakdown product was identified as glucose-1-phosphate (Cori-ester). It could then be demonstrated that from this substance—but not from glucose!—an enzymatic synthesis of both glycogen and starch is possible (133).

Similarly, Lipmann found that the oxidation of pyruvic acid, proceeding by way of phosphopyruvic acid, yields, in addition to CO_2 and a reduced hydrogen acceptor, acetyl phosphate instead of acetic acid as a primary product. Later it was established that pyruvic acid can be synthesized enzymatically from acetyl phosphate, CO_2 , and hydrogen (134). The recent studies of Barker (135) have further emphasized the importance of acetyl phosphate—as contrasted with acetate—for the synthesis of fatty acids. The latter are formed by the enzymatic condensation of a lower fatty acid with acetyl phosphate and a subsequent reduction of the β -keto acid.

The last-mentioned process is a good example of a special type of reversible reaction in which fragments of molecules, sometimes of considerable size, are transferred to other molecules. Here belong those reactions known as trans-methylations, trans-aminations, trans-phosphorylations, and trans-glucosidations (76, 134, 136, 137).

With these few general ideas it is at present possible to account for the synthesis of monosaccharides, polysaccharides, fatty acids,

and amino acids as the result of various series of relatively simple and chemically intelligible step reactions. This is exactly the fundamental concept expressed many years ago by Kluyver. But the details concerning the true nature of the steps have meanwhile undergone important modifications which make it possible to consider the problem of the primary product of photosynthesis and of oxidative and fermentative assimilations from a broader point of view.

A survey of present-day knowledge of biosynthetic mechanisms reveals that a small number of compounds can serve in a variety of reactions. A striking example is pyruvic acid. By a reversal of the glycolytic mechanism this substance, or its phosphorylated derivative, may yield carbohydrate or hexosephosphate. It may also, by an oxidative decarboxylation, produce acetyl phosphate, and in this manner represent a potential starting point for fatty acid synthesis. Through addition of ammonia it is readily convertible into an amino acid. Or, again, it can act as a CO_2 acceptor and thus give rise to the dicarboxylic acid series of compounds. In all these reactions a reduction accompanies the transformations; hence it is understandable that processes of hydrogen transference are indispensable for any and all of these syntheses. However, and this is the important conclusion, it is not imperative that pyruvic acid be exclusively converted by only one specific route under the influence of an organism. It is far more likely that, depending upon internal and external factors, several of these reaction chains will occur simultaneously, although one or the other may be predominant.

As has been pointed out above, there is much evidence to favor the hypothesis that the assimilation of CO_2 in photosynthesis is accomplished in a dark reaction through a reversal of some decarboxylation, and that the Krebs tricarboxylic acid cycle offers reasonable possibilities for such reactions. Now it is in this very cycle that the key compounds for several fundamental biosyntheses can be found. It consequently follows that the above considerations may well apply to photosynthesis. This leads to a very flexible concept concerning the formation of organic matter by this process, *viz.*, that in view of the nature of the initial products of CO_2 fixation a number of pathways exist for their immediate transformation into a variety of substances. It is quite possible that different photosynthetic organisms have developed in such a manner that some special routes have become quantitatively predominant. But potentially all of the now known conversions might be involved.

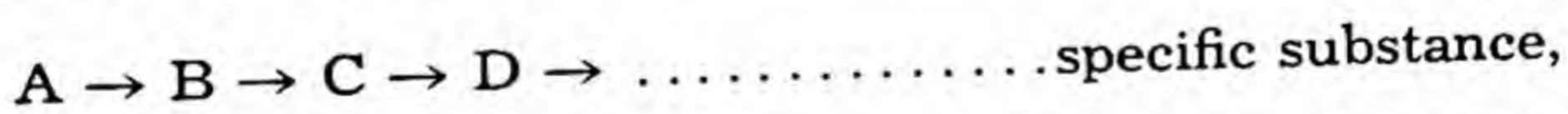
It need hardly be mentioned that the same supposition can be made regarding the oxidative and fermentative assimilations.

Although the above general outline of present-day knowledge of biochemical syntheses has many attractive features, it is advisable to bear in mind its sketchy nature. But it has the advantage, inherent in any sketch, that details may be fitted in as the occasion demands, and at this time it appears to me more useful than a rigid scheme for which compelling evidence is wanting.

No matter how impressive has been the advance in our understanding of biosyntheses, there are still many and important problems in this field awaiting a solution. Nothing much is known, for example, concerning the mechanisms whereby such fundamental structures as the benzene, pyridine, pyrrol, pyrimidine, or thiazole rings are synthesized. Nor is any pertinent information available regarding the mode of formation of carotenoids, purines, and most other groups of complex substances. In spite of these enormous gaps the prospects are encouraging, and it may be confidently expected that future studies will soon reduce these inadequacies in our knowledge.

Two methods of approach, both in current use, seem particularly appropriate for attempting a solution of such problems. The first is based directly upon the reversibility principle, and consists of a detailed study of the stepwise biological degradation of the compound whose mode of synthesis is to be investigated. The various microorganisms capable of decomposing such substances offer the best prospects for this line of attack.

The second has been used successfully for determining the mechanism of synthesis of those substances which are essential components of the organism. Obviously, an organism incapable of synthesizing a necessary cell constituent from the nutrients provided by the medium cannot grow unless supplied either with the compound itself, or with materials from which it can synthesize that substance. On the assumption that the synthesis proceeds by an orderly series of steps:



an analysis of this series can be carried out if the addition of possible precursors results in growth. The most significant advances made by this method have resulted from the demonstration that the synthetic abilities of an organism can be modified by inducing mutations. The epoch-making studies of Beadle and Tatum and their collaborators (138) have shown that, in general, the physiological mutants differ from the parent strain by the incapacity of the former to perform one single step in some synthetic mechanism, and, furthermore, that any one step in the series may be

blocked in the mutated offspring. Hence the investigator is in a position to start with an organism which can accomplish the synthesis of a particular compound from simple and only distantly related substrate molecules. Such an organism consequently must be able to catalyze a large number of steps all of which are involved in the synthetic mechanism. By inducing mutations it is possible to obtain variants unable to grow in the original medium because they can no longer synthesize the substance in question from the materials supplied; theoretically it would even be possible to produce mutants that lack the capacity to carry out any one of the steps of the many synthetic mechanisms which the initial culture possessed. A study of the effect which various precursors exert on the growth of those variants thus makes it possible to reconstruct the series of reactions involved in the synthesis of the specific substance.

For neither of these two methods of approach is it necessary to use photosynthetic organisms. And it is the contention of the foregoing pages that a better understanding of the biosynthetic activities of this group can certainly be anticipated as a result of studies with non-photosynthetic organisms. Of course, this does not imply that all biosyntheses of plants and photosynthetic bacteria can be unraveled with the aid of mutants of other types of organisms; it is quite possible that some substances are characteristic products of photosynthesizing cells only. The formation of the group of chlorophyllous pigments may well be a case in point, since these substances have not been reported in other organisms. It should also be emphasized that there is unequivocal evidence for the occurrence of specifically photochemical reactions which are responsible for the integrated, normal development of plants. The phenomenon of photoperiodicity provides a striking example of such reactions. Even the formation of chlorophyll might be included here, since it is known that, at least in the higher plants, this process is dependent upon proper illumination. However, the light intensities involved in these cases are so extremely low that this fact alone provides a sufficiently strong argument for the viewpoint that such photochemical reactions cannot be primarily concerned with the mass-production of organic matter or with the major transformations of the latter, but at best with the manufacture of trace quantities of particular compounds possessing a regulatory activity. A further discussion of such specialized phenomena falls outside the scope of the present essay, whose chief function it is to indicate the application of the principles of comparative biochemistry to the major aspects of photosynthesis.

PHOTOSYNTHESIS AND ORGANIC EVOLUTION

"Après une mure délibération, les auteurs du présent ouvrage se sont mis d'accord pour considérer comme impossible l'apparition primitive d'organismes autotrophes, bien que ce soit là un postulat admis, sinon comme plausible, du moins inéluctable."—A. DAUVILLIER AND E. DESGUIN.

In the foregoing pages the main problem of photosynthesis, *viz.*, the formation of organic matter from CO_2 by specialized living organisms under the influence of light, has been treated as a problem in comparative biochemistry. As mentioned before, such treatment rests on the assumption that all living organisms effect the general transformation of matter by fundamentally similar mechanisms.

Experimental evidence in favor of this viewpoint is not lacking and special cases have already been cited. The most compelling arguments have, however, so far not been mentioned; they are derived from the recent studies on enzymes, vitamins, and amino acids.

It has been firmly established that the fundamental enzyme systems, such as the phosphopyridine, and the alloxazine enzymes, the carboxylases, decarboxylases, phosphorylases, etc., can be found in all living organisms with the exception of that group which lies on the borderline between the conventionally defined living and non-living world, the viruses. This is further supported by the universal occurrence in living organisms of the water-soluble vitamins, now recognized as parts or immediate precursors of enzyme systems. Indeed, many a biochemist nowadays is approaching the study of enzyme systems through investigations of vitamin requirements, and with promising results.

In a large measure this has been due to important developments in the field of nutrition. It has now become a basic tenet that the need of an organism for certain dietary factors carries a twofold implication. First, it means that those substances are used for the manufacture of essential cell constituents; second, that such constituents cannot be synthesized by the organism—either at all, or at a sufficiently high rate—from other components of the diet. When applied to factors, such as vitamins, which are required in minute amounts, this concept leads to the conclusion that they are transformed into compounds of high activity so that only a relatively small supplement is enough to satisfy the needs of the cell. Hence their connection with the biocatalysts, or enzyme systems, becomes obvious.

A further consequence is that an organism that does not require one or more of those dietary factors should be able either to function normally through mechanisms of which they do not constitute a part, or to synthesize them from other substances in the diet. So far, the evidence with respect to the B-vitamins is overwhelmingly in favor of the latter alternative.

Similar conclusions can be drawn from the results of studies on amino acid nutrition. Here, too, the available evidence endorses the view that some twenty amino acids are fundamental constituents of all living cells, and that organisms capable of growing without being supplied with one or more of these substances can do so only because they manufacture the missing members from other foodstuffs.

This universal occurrence of the same amino acids, vitamins, and enzyme systems in all living organisms is the most potent argument today for a monophyletic origin of life. The developments in the field of biochemistry thus supplement the evidence derived from morphological and anatomical studies in the previous century. They also suggest new possibilities for an approach to the problem of evolution.

When biochemical considerations were first introduced into discussions of this problem, it was tacitly assumed that life on earth had its start in a strictly mineral environment. Consequently the "first organisms" should have been capable of producing organic matter from inorganic. It was conceded that the question as to whether chemo-autotrophic had preceded photoautotrophic organisms or *vice versa* was hard to decide. The fact that so many organisms require oxygen, and the only known source of this gaseous constituent of our atmosphere is the photosynthetic process of green plants, indicated that they must have been among the earliest inhabitants of the earth. The requirement for organic foodstuffs branded the other types of organisms as latecomers, and an increase in the complexity of their nutrient requirements was taken to indicate an increased tendency toward specialization and an advanced stage of evolution.

In an excellently documented treatise Orla-Jensen (139) used this line of reasoning as early as 1909 to trace the evolution of the various types of bacteria. A considerable amount of work has since been published which tends to support the Danish master's contentions. Especially André Lwoff (140) has ably defended the thesis that the present-day colorless and plastidless protozoa are derived from plastid-containing and chlorophyll-bearing ancestors, more especially the phytomonads. This is reflected in the clearly discernable relationships between the colorless phytomonads with

leucoplasts and their pigmented relatives, as illustrated in the groups *Euglena-Astasia*, *Chlamydomonas-Polytoma*, and others. The physiological effect of the loss of chlorophyll is that the organism can no longer live by means of photosynthesis and now becomes dependent upon a supply of preformed organic matter. In the final analysis the latter means dependence upon other living organisms, which in turn, has resulted in the loss of power for the synthesis of a variety of essential cell constituents, such as vitamins. These adaptations, or evolutionary changes, in the groups of protozoa—and this is the group mainly considered by Lwoff—are therefore clear-cut examples of a gradual tendency towards the need for an increasingly complex environment, and physiological evolution can be regarded as the consequence of a series of losses in synthetic ability.

This thesis has gained much support from the earlier mentioned studies of Beadle *et al.* (138), demonstrating that the artificially produced physiological mutants so far investigated differ from the parent organism in having lost the ability to perform some step in a biosynthetic sequence. Corresponding results have recently been obtained in similar studies on bacteria by Tatum and collaborators (141).

The unavoidable consequence of the adoption of this point of view seems, however, very difficult to accept. It is that the earliest, most primitive living organisms must have been endowed with a complete set of biosynthetic mechanisms. If evolution, on a physiological plane, has operated only through the consecutive loss of enzyme systems (or of genes) which control the numerous steps of biosynthetic processes, then the ultimate ancestor of present-day living beings must have been endowed with a full complement.

It is against this consequence that Oparin (142), several years ago, voiced a well-documented opposition. From a physico-chemical standpoint the origin of such an organism would be nothing short of miraculous. What we call a living cell represents far more than a vast complexity of organic compounds; it also implies their integration into a harmonious entity capable of self-reproduction. The spontaneous emergence of such a system in an inorganic world, without any simpler precursors, could be accounted for only on the basis of a special act of creation.

In order to advance a scientifically more acceptable approach, Oparin postulated an origin of life, not in an inorganic environment, but in one which abounds in a great diversity of organic molecules, including the most complex kinds. The existence of such a system, as yet devoid of living organisms, is regarded as the result of

chemical interactions between simple organic molecules, such as hydrocarbons, which have been detected in the atmospheres of stellar bodies. Due to gradually changing conditions the more elaborate structures could, in the course of time, persist for sufficiently long periods to give rise to ever more complicated ones, so that in the end all the essential constituents of what we now consider a living organism would be present. The eventual combination, through chance-associations, of such compounds could then produce an entity with some properties of a living cell. However, this is not the sort of system which is endowed with complex synthetic abilities; on the contrary, its powers of synthesis need only be at a minimum, since the environment in which it originates contains all the constituents in a preformed state. It is important to note that Oparin has emphasized that the existence of a world in which the requisite variety of organic molecules can originate is not only consistent with, but actually dependent upon the initial absence of living organisms. Oparin's ideas can also be found in an essay by Haldane (143, pp. 145-56) on "The origin of life," evidently written as early as 1928, and containing the following significant passages:

"Now, when ultraviolet light acts on a mixture of water, CO_2 , and ammonia, a vast variety of organic substances are made, including sugars and apparently some of the materials from which proteins are built up.....In the present world such substances, if left about, decay—that is to say, they are destroyed by microorganisms. But before the origin of life they must have accumulated till the primitive oceans reached the consistency of hot dilute soup. Today an organism must trust to luck, skill, or strength to obtain its food. The first precursors of life found food available in considerable quantities, and had no competitors in the struggle for existence" (p. 152). And it appears that Dauvillier and Desguin (144), and Beutner (145) have independently arrived at very similar conclusions.

Such a view of the spontaneous generation of an organism with extremely limited synthetic powers is not only plausible; it can also be used, as Horowitz (146) has pointed out in a highly important contribution, to account for the genesis of biosynthetic mechanisms of the most complex nature. Because the synthesis of even a simple amino acid requires the sequential operation of an extended series of enzymes and genes, one is faced with the difficulty of envisaging the gradual emergence of such reaction chains and of the catalysts under whose influence they proceed. It is evident that the absence of a single link in the chain would imply

an utter uselessness of the rest of its components. Hence it has been argued that it is virtually impossible to comprehend the acquisition of biosynthetic mechanisms on the basis of probability. This difficulty can, as Horowitz has logically demonstrated, be overcome if one postulates that the chains are developed, not by the stepwise production of enzymes which carry out the synthesis starting at the inorganic level, but by the emergence of such enzyme systems, one at a time, in a reverse sequence. In this event only the last step of the chain represents a new acquisition which, in addition, has "survival value." And this again can be achieved by a simple, single-gene mutation.

In this manner the Haldane-Oparin-Horowitz hypothesis can serve to surmount the fundamental difficulties inherent in the major problem of biological philosophy: that of the origin of life. It solves this enigma by assuming a physiological evolution in which the mechanisms for the synthetic reaction chains are gradually acquired, instead of lost. Admittedly, the evidence adduced by Lwoff in support of his antithetical concept, as well as the results of the studies of Beadle, Tatum, *et al.*, cannot be lightly dismissed. It will be necessary to accept the occurrence of a physiological evolution characterized by a loss of synthetic ability, and it is even probable that this is the chief if not the exclusive route by which the vast majority of now living organisms have originated. But acceptance of this view does not rule out the possibility that the earliest stages of physiological evolution have been marked by events along the lines suggested by Haldane and Oparin, and this seems to me the most plausible hypothesis.

In that case the chemo- and autotrophic organisms would represent advanced rather than early stages of physiological evolution, and, on account of their greater biosynthetic achievements, the photosynthetic organisms should even be considered as the most highly developed biochemical types. Such a conclusion was also reached by Blum (147) in an admirable attempt to outline the evolution of photosynthesis.

Blum's line of reasoning was, however, different from the one presented here. It was based upon the difficulty of conceiving the genesis of photosynthesis as a *multiquantum photochemical process* other than by successive stages. The suggestion was made that photosynthetic organisms had evolved from chemo-autotrophic ones (type A), as follows:

"In the earliest form of photosynthesis (type B) the light quantum may have served only to supply energy of activation and thus to speed up a reaction which could go spontaneously without the

addition of energy, as is the case for many *in vitro* photochemical reactions. Later, organisms may have evolved in which carbohydrate or other organic material was formed by some reaction in which only one quantum was necessary to provide the energy to forward a reaction which would not go spontaneously (type C). From these systems, organisms might have evolved which used two or three quanta (type D) until chlorophyll photosynthesis requiring four quanta (type E) was finally evolved" (147, p. 356).

In view of the energy changes in photosynthesis of the green bacteria ($\Delta F = +18,000$ cal.; $\Delta H = +22,000$ cal.), it is thermo-dynamically possible that this type of reaction might be accomplished with only one quantum of light (40,000 cal. at 700 m μ .), and it was therefore assumed that these bacteria might represent "primitive" photosynthetic organisms of type C, or their descendants. The purple bacteria, requiring an energy supply corresponding to a minimum of one to three light quanta, might then represent further evolutionary stages (type D) or their derivatives.

Unfortunately, quantum yield measurements have not yet been reported for green bacteria photosynthesis. And the best ones published for purple bacteria photosyntheses (148, 149) indicate strongly that here, too, far more energy must be supplied as light quanta than would appear necessary on purely thermodynamic grounds, just as has been found for green plant photosynthesis in the years following Blum's publication. It might, of course, be objected that the metabolism of present-day photosynthetic organisms, perhaps greatly modified from that of the primitive types, cannot legitimately be used to argue against the validity of this hypothesis. There is, however, another reason for approaching the problem of the evolution of photosynthesis in a different manner.

I have endeavored in this essay to show that there are good grounds for postulating that the photochemical reaction in the photosynthetic processes of green bacteria, purple bacteria, and green plants represents, in all cases, a photodecomposition of water. If this be accepted, photosynthesis cannot any longer be considered as a multiple quantum reaction in the same sense in which this designation was employed by Blum. Each quantum hit could produce one photolytic reaction, *i.e.*, one transferable hydrogen atom, and the summation of the quantized energy thus becomes the result of enzyme reactions concerned with hydrogen transfer. Such reactions are not in themselves photochemical, and the responsible enzyme systems might, for example, be found also in the nitrifying or hydrogen oxidizing chemo-autotrophic bacteria. It seems reasonable to assume that the genesis of these enzyme systems has been a phase in the physiological evolution of the non-photosynthetic

organisms. The latter would thereby become ever less dependent upon a supply of organic substances.

After the development of a mechanism for CO_2 assimilation in a non-photosynthetic organism, a further evolutionary step would consist in the acquisition of an appropriate pigment system capable of provoking the photodecomposition of water. In this manner photosynthetic organisms could have emerged. But I find it difficult to imagine that the first photosynthetic organisms would have been able to carry out the type of process which is now so characteristic of green plant photosynthesis, because the latter exhibits not one, but two radically new features that distinguish it from the metabolism of non-photosynthetic organisms endowed with the ability to assimilate CO_2 . They are; (a) the existence of a photochemical mechanism, and (b) the liberation of molecular oxygen.

Hence it seems logical to attempt a reconstruction of the evolution of photosynthesis in such a way that only one of these two features is added at a time to the mechanisms operating in the non-photosynthetic organisms. The ability to produce molecular oxygen has not yet been encountered in any organism except the green plants. On the other hand, photosynthesis without oxygen production is precisely what characterizes the metabolism of the green and purple bacteria. These organisms may therefore be considered as representing the sought-for intermediate stage in the physiological evolution of green plant photosynthesis. The photosynthetic bacteria could be derived from non-photosynthetic organisms by the gradual development of a pigment system capable of inducing a photodecomposition of water. The photolysis yields, however, in addition to the hydrogen which is used for CO_2 reduction, an oxidized component, and the latter must be reduced before it can again participate in the photochemical reaction mechanism. This process requires the presence of extraneous reducing compounds, and the operation of previously acquired enzyme systems which catalyze the transfer of hydrogen from the external donor to the oxidation product.

Once the mechanism for the photodecomposition of water had been developed, further evolution would depend upon the occurrence of modifications whereby the oxidized component of the photochemical mechanism no longer requires reduction with the aid of external hydrogen donors, but becomes capable of self-regeneration through the elimination of molecular oxygen. This, then, represents the final step in the emergence of green plant photosynthesis.

Interpreted in this manner, green plant photosynthesis appears as the ultimate result of that line of physiological evolution which

is characterized by the gradual development of synthetic mechanisms. The first of these mechanisms came into being in an environment containing a multitude of organic compounds, and operated through the catalysis of a consecutive series of dark reactions. The succession of organisms arising during this phase marked a trend towards increasing independence of the supply of organic matter. The chemo-autotrophs represent the final stage of this evolutionary tendency; these organisms can dispense with organic substrates altogether. They are, nevertheless, still dependent upon the presence of oxidizable, though inorganic, substances.

With the appearance of pigment systems capable of performing a photodecomposition of water, the first step was taken towards independence from extraneous hydrogen donors, since the water molecule itself could be used as a source of hydrogen for the reduction of CO_2 . The end result of this line of development was the evolution of the green plants. Here we find the trend carried to its extreme conclusion. These forms of life have achieved complete emancipation; they can develop in an environment devoid of all oxidizable matter.

Physiological evolution, insofar as it pertains to the progressive development instead of the loss of function, can thus be paraphrased as the stepwise acquisition of complete independence from external reducing substances.

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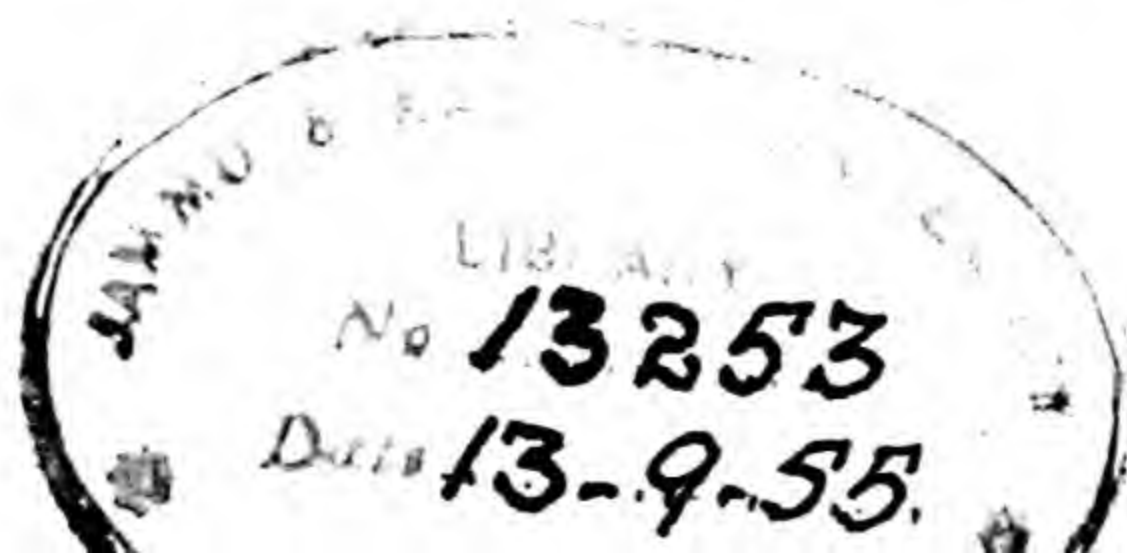
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